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Expanding the Use of Electroporation from Cutaneous to Intraluminal and Systemic Applications

Thesis presented by

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Table of Contents

Declaration

I certify that this thesis has not been previously submitted for a degree to this or any other University. This thesis is the result of my own investigation and any other assistance is acknowledged. I authorize University College Cork to lend and photocopy this thesis to other institutions or individuals for the purpose of scholarly research.

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Mira Sadadcharam

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Finally, I dedicate this thesis to my beloved grandparents who taught me the meaning of unconditional love, who paved the path before me and upon whose shoulders I stand.

Index of Abbreviations

APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
B-gal	β-Galactosidase
bp	base pairs
cDNA	Complementary Deoxyribonucleic Acid
CR	Complete Response
CTL	Cytotoxic T Lymphocytes
DMEM	Dulbecco's Modified Essential Medium
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dsRNA	Double-stranded Ribonucleic Acid
ECT	Electrochemotherapy
EDTA	Ethylenediamine Tetraacetic Acid
EGT	Electrogenetherapy
ELISA	Enzyme-linked Immunosorbent Assay
ESOPE	European Standard Operating Procedures for Electrochemotherapy and Electrogenetherapy
FACS	Fluorescent-Activated Cell Sorter
Gm-CSF	Granulocyte Macrophage Colony-Stimulating Factor
Hz	Hertz
i.m.	Intramuscular

i.p.	Intraperitoneal
i.v.	Intravenous
IU	International Units
Kb	kilobase pairs in duplex nucleic acid
mAb	Monoclonal antibody
mRNA	messenger ribonucleic acid
nsP	Non-structural Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute
s.c.	subcutaneous
SOP	Standard Operating Procedure
TCR	T cell Receptor
TGFβ	Transforming Growth Factor beta
TIL	Tumour Infiltrating Lymphocytes
Treg	T Regulatory Cell
TUNEL	Transferase-mediated dUTP nick end-labeling
US	Ultrasound
V	Voltage

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1. Electrochemotherapy: an emerging cancer treatment.Sadadcharam M, Soden DM, O'sullivan GC. Int J Hyperthermia. 2008 May;24(3):263-73. doi:10.1080/02656730701832334. Review. Erratum in: Int J Hyperthermia.2008 May;24(3):273. PubMed PMID: 18393004.

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3. Enhancement of electroporation facilitated immunogenetherapy via Treg depletion. Forde PF, Sadadcharam M, Hall LJ, O' Donovan TR, de Kruijf M, Byrne WL, O'Sullivan GC, Soden DM. Cancer Gene Ther. 2014 Aug;21(8):349-54. doi:10.1038/cgt.2014.35. PubMed PMID: 25034887

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Presentations

International Presentations

1. October 27th – 30th 2007: European Society of Gene and Cell Therapy, Rotterdam: "Application of electroporation-driven intraluminal gene delivery." M.Sadadcharam, P. Forde, L. Cogan, D. Soden, G.C. O'Sullivan

2. January 9th – 11th 2008: Society of Academic & Research Surgery Annual (SARS) Meeting, Birmingham: "Clinical utility of electroporation-mediated intraluminal gene delivery." M.Sadadcharam, P. Forde, L. Cogan, D. Soden, G.C. O'Sullivan

3. May 30th – June 3rd 2007: American Society of Gene Therapy's 10th Annual Meeting, Seattle: "An Endoscopic Tool for Targeted Gene and Drug Delivery." Declan M. Soden, Mira Sadadcharam, John Piggott, Timothy Doody, Gerald C. O'Sullivan

4. June 26th – 30th 2007: 11th Mediterranean Conference on Medical and Biomedical Engineering and Computing 2007 (MEDICON), Slovenia: "An endoscopic system for gene and drug delivery to intraluminal tissue. Declan Soden, Mira Sadadcharam, John Piggott, Anthony Morrissey, C.G.Collins, G.C. O'Sullivan

National Presentations

1. September 7th – 8th 2007: 32nd Sir Peter Freyer Memorial Lecture and Surgical Symposium, Galway: "Application of Electroporation-Driven Intraluminal Gene Delivery." M. Sadadcharam, J. Piggott, D. Soden, G.C. O'Sullivan

2. March 2nd – 3rd 2007: Irish Association of Cancer Research, Cork: "Application of Endoscopic Electrochemotherapy." M. Sadadcharam, J. Piggott, D. Soden, G.C. O'Sullivan

3. May 18th 2007: Irish Society of Gene and Cell Therapy, Galway: "Application of electroporation gene delivery to cutaneous and subcutaneous metastatic lesions." M. Sadadcharam, J. Piggott, D. Soden, G.C. O'Sullivan

4. November 13th – 4th 2008: Irish Society of Gastroenterology, Meath "Endoscopic Gene and Drug Delivery for Gastrointestinal Tissues and Tumours – A New

Development.”. M. Sadadcharam**,** P. Forde, M. de Kruijf, L. Cogan, J.O. Larkin, C.G. Collins, D.M. Soden, G.C. O’Sullivan

Chapter 1

Introduction

1.0 The Burden of Cancer

Cancer is a global phenomenon transcending the boundaries of age, race, geography and socioeconomic background. Today, cancer accounts for about 1 in 7 deaths worldwide – more than HIV/AIDS, malaria and tuberculosis combined [1]. In 2012, there were an approximately 14.1 million cases of cancer diagnosed worldwide with 8.2 million cancer-related deaths. In addition, the global cancer burden is growing at an exponential pace; in 2030 alone, approximately 21.7 million new cancer cases and 13.0 million cancer deaths are expected to be diagnosed [1].

The causes of intractability include advanced stage disease at presentation which limits the application or effectiveness of treatments, resistance of tumour cells to chemotherapy and radiation therapy, anatomical locations of the cancer that preclude complete excision or ablation by radiotherapy, and the presence of intercurrent illnesses which either limit the application or adversely alter the risk-benefit profile of any given treatment [2-6].

1.1 Conventional Cancer Therapies

The ideal treatment of cancer should effectively control local disease, be applicable to a diversity of tumour types and anatomical locations, be effective against locally recurrent disease, facilitate multimodal and systemic therapies, be minimally intrusive and improve patient wellbeing and life expectancy by tumour control or cure.

The most effective treatment strategies are those which provide healthcare in a sustained and equitable fashion, are linked to early detection, and which adhere to evidence-based standards of care and a multidisciplinary approach. The principal methods of treatment are surgery, chemotherapy (including hormonal manipulation) and radiotherapy. Although each has a well-established role in its own right and can cure certain types of cancers, multidisciplinary management is more effective than sequential independent management of patients [7-11].

1.1.1. Surgery

Modern medicine has seen a move away from interventions of the past which were ‘primarily surgical, primarily heroic, and primarily unsuccessful’. The field of surgical oncology has emerged as a specialist discipline with recent advances including more precise identification of tumour margins, leading to reduced local

recurrence. However, while surgical resection has proven to be effective in the control of primary tumours and early-stage disease, its contribution to the management of regional or metastatic disease is significantly attenuated [12-17].

1.1.2 Chemotherapy

Despite the existence of an array of chemotherapeutic agents, cancer survival rates for clinically advanced malignancies have improved little during the past three quarters of a century. Among the limitations of conventional chemotherapy is the lack of tumour sensitivity to existing chemotherapeutic regimens, as well as the development of drug resistance. The mechanisms responsible for this refractivity include a reduction in drug uptake, enhanced drug export, intracellular inactivation of the effective agent, alteration of the molecular target, an increase in the activity of the target route to be inhibited or the appearance or stimulation of alternative routes, enhanced repair of drug-induced modification in the target molecules, and activation/inhibition of intracellular signalling pathways, which leads to a negative balance between apoptosis/survival of tumour cells [18-24].

1.1.3 Radiation therapy

Radiation therapy has been in use as a cancer treatment for more than 100 years. It has been estimated that between 50-60% of cancer patients will benefit from radiotherapy. The major indications for radiotherapy in anti-cancer strategies include head and neck cancers, gynaecological cancers (e.g. cervix), prostate cancer, pelvic malignancies (e.g. rectum, bladder), brain cancers, and as adjuvant therapy in breast cancer [25]. Radiotherapy is also routinely used in a palliative context, to reduce pain and address acute symptoms such as spinal cord compression.

While radiation therapy in itself is painless, it is associated with a significant side-effect profile. Although most radiotherapy side-effects are predictable, the nature, severity, and longevity of side effects depends on the organs that receive the radiation, the treatment itself, and the patient [26-30].

1.2 The Need for Novel Therapeutics

The more we discover of the molecular diversity of tumours, the more we realise that cancer therapies need to be engineered towards molecularly-defined subsets of tumours [31]. Moreover, the advent of more targeted molecular therapies has been countered by the ability of the cancer cell to mutate or employ epigenetic disguise.

There is also little doubt that it is in the transition from laboratory to clinic where most is lost in translation.

There is now renewed optimism that many cancers can be cured or forestalled by immune-based therapies used either alone or as part of multimodal programmes [32-36]. This has resulted from an improved understanding of tumour immune interactions, and the viability of cell, gene and ligand-based technologies to promote effector anti-tumour immune responses.

In this chapter, I will examine the literature reporting research into the conceptualisation and development of electroporation and systemic gene and immunotherapy anticancer treatments since these areas are directly relevant to my work. For each of these therapeutic modalities, I will explore both their present utility and the current limitations to their clinical application.

1.3 Electroporation

1.3.1 Principle

The cell membrane represents a physical barrier to the intracellular transfer of hydrophilic drugs, macromolecules, nucleotides and peptides. The phospholipid bilayer of the plasma membrane restricts the transmembrane movement of hydrophilic and polar molecules, other than by specific channel or receptor-mediated assistance. The application of electric fields has been utilized to overcome this barrier and permit the traffic of various molecules. This strategy is referred to as electroporation. Short high-voltage electric field pulses, optimised to balance the reversible and irreversible effects of the procedure [37, 38] (Figure 1.1) are used to render the cell membrane transiently permeable or porous [37, 39]. Using this technique, a wide range of molecules, e.g., ions, peptides, oligonucleotides, RNA and DNA and hydrophilic drugs have been successfully delivered intracellularly [40-43].

1.3.2 Pulse parameters

In clinical practice, optimised pulse parameters (8 square wave pulses; amplitude 1300 V/cm; duration 100 ms; frequency 1Hz) are utilised to permeabilise tumour cell membranes (Figure 1.1). Using these pulse parameters, drug dosages that have minimal or no anti-tumour effectiveness demonstrate high (75%) complete response rates in treated tumour nodules [44-46].

1.3.3 Pulse generator

A number of pulse delivery systems are commercially available including the Cliniporator (IGEA) and the MedPulser® (Inovio). However, for the purposes of this thesis, the ePORE Gx was used for optimised pulse delivery (Figure 1.2).

1.3.4 Electrode systems

The electric field distribution is largely determined by the geometry of the electrodes and these can be divided into two different types: plate electrodes and needle electrodes (Figure 1.3). Plate electrodes are suited for use in skin or superficial lesions. Needle electrodes are either arranged in two parallel rows or in a circular (hexagonal) array. Unlike plate electrodes, these are impaled transcutaneously into the target tissue. Regardless of the type of electrode, the electric field is highest around and between the electrodes. In addition, different tissue types will have varying degrees of tissue pliability and electrical conductivity. This needs to be taken into consideration when deciding which electrode design to use in any given situation.

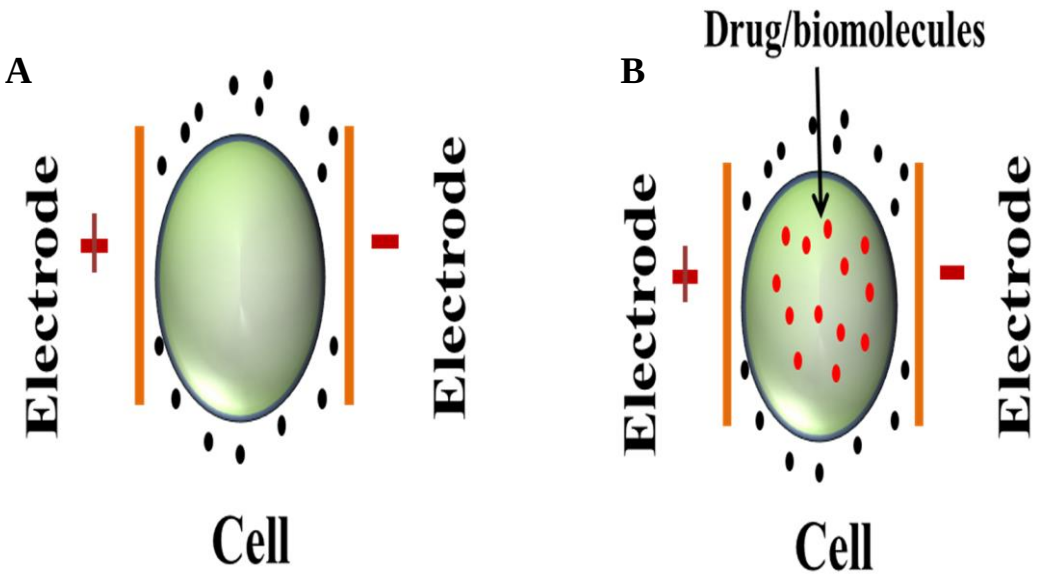

Figure 1.1: **(A)** Electric field applied to the cell membrane; **(B)** When external electric field reaches a threshold value, the cell membrane is permeabilised to deliver drug/biomolecules inside the single cell. After electroporation, the cell membrane reseals again [47].

Figure 1.2: The ePORE Gx, clinically approved pulse generator used to deliver permeabilising electric pulses.

Figure 1.3: (Top: L – R) Plate electrodes; hexagonal array needle electrodes; parallel array needle electrodes. (Bottom: L – R) EndoVe, a novel electrode system designed for delivery of electroporating pulses to gastrointestinal tissues and tumours; ThoraVe, a novel electrode device designed for delivery of electroporating pulses to pulmonary tissues and tumours. The choice of electrode used for electropermeabilisation is influenced by a number of factors, including tumour locale, the pliability of the tissue to be electroporated, the likelihood of encountering resistance (e.g. previously irradiated skin) and physician preference.

1.3.5 Electrochemotherapy

Electrochemotherapy involves the local application of pulses of electric current to tumour tissue rendering the cell membranes transiently permeable to otherwise impermeant or poorly permeant anticancer drugs such as Bleomycin or Cisplatin, thereby facilitating a potent localised cytotoxic effect.

Studies have been performed in a wide variety of tumour types, including melanoma, hepatocellular carcinoma, lung carcinoma, breast carcinoma, fibrosarcoma, and glioma [48-52]. The cytotoxicity of Bleomycin has been shown to be increased by up to 700-fold resulting in objective response rates as high as 90%.

The ESOPE study (European Standard Operating Procedures of Electrochemotherapy) was a prospective, non-randomised multi-centre project funded under the European Commission's 5th Framework Programme. They assessed treatment outcomes following electrochemotherapy based on tumour histology, chemotherapeutic agent, route of administration and electrode type. Patients were recruited from four centres, namely: Institute Gustave-Roussy (IGR), Villejuif, France, Institute of Oncology Ljubljana (OI), Ljubljana, Slovenia, University of Copenhagen at Herlev Hospital (HH), Herlev, Denmark, and the Cork Cancer Research Centre Biosciences Institute and Mercy University Hospital, National University of Ireland (CCRC), Cork, Ireland. Electrochemotherapy with Bleomycin revealed an objective response rate of 85% (73.3% complete response rate) [45, 53]. Electrochemotherapy with Cisplatin resulted in 77% long lasting complete responses of tumour nodules. This held true over a variety of tumour histologies, drugs and routes of administration. There was also a trend towards higher anti-tumour activity in non-melanoma nodules (OR 90.4% versus 80.6%).

Electrochemotherapy is now used in the treatment and palliation of a wide histological variety of tumours. The encouraging clinical experiences to date promise the development of systems for treatment of intractable deep-seated malignancies [54-62].

1.3.6 Present Limitation of Clinical Utility

Until recently, the full clinical utility of electroporation has been restricted due to limitations in electrode design. Modification of existing designs to allow for attachment to conventional endoscopic and laparoscopic equipment would allow for the clinical license of this therapy to be greatly extended [63]. If it were possible to

deliver permeabilising electric pulses to luminal tumours e.g. intra-abdominal, intra-thoracic and genitourinary malignancies, tumours that would previously have been unresponsive or inaccessible to conventional therapies would be amenable to electrochemo- or electrogene therapies.

In this thesis, we will describe the development and validation of an endoscopic electrode device, the EndoVE, designed to extend the clinical utility of electroporation to the management of luminal tumours. If this system proves successful, many otherwise intractable or inaccessible tumours would be rendered amenable to treatment.

1.4 Cancer Gene Therapy

In 1989, Rosenberg *et al* performed the first human gene therapy trial when they used a retrovirus to introduce the gene coding for resistance to neomycin into human tumour-infiltrating lymphocytes before infusing them into five patients with advanced melanoma [64]. Since then, over 1700 clinical trials have been completed, are ongoing or have been approved for use worldwide [65-70].

1.4.1 Present Utility of Cancer Gene Therapy

The aim of gene therapy is to introduce a defined DNA sequence into specific cells in order to replace a defective gene, or to impart a new function to the cell in order to induce it to secrete a therapeutically-functional protein. In practise, Figure 1.4 demonstrates the diseases in which gene therapy has been approved so far.

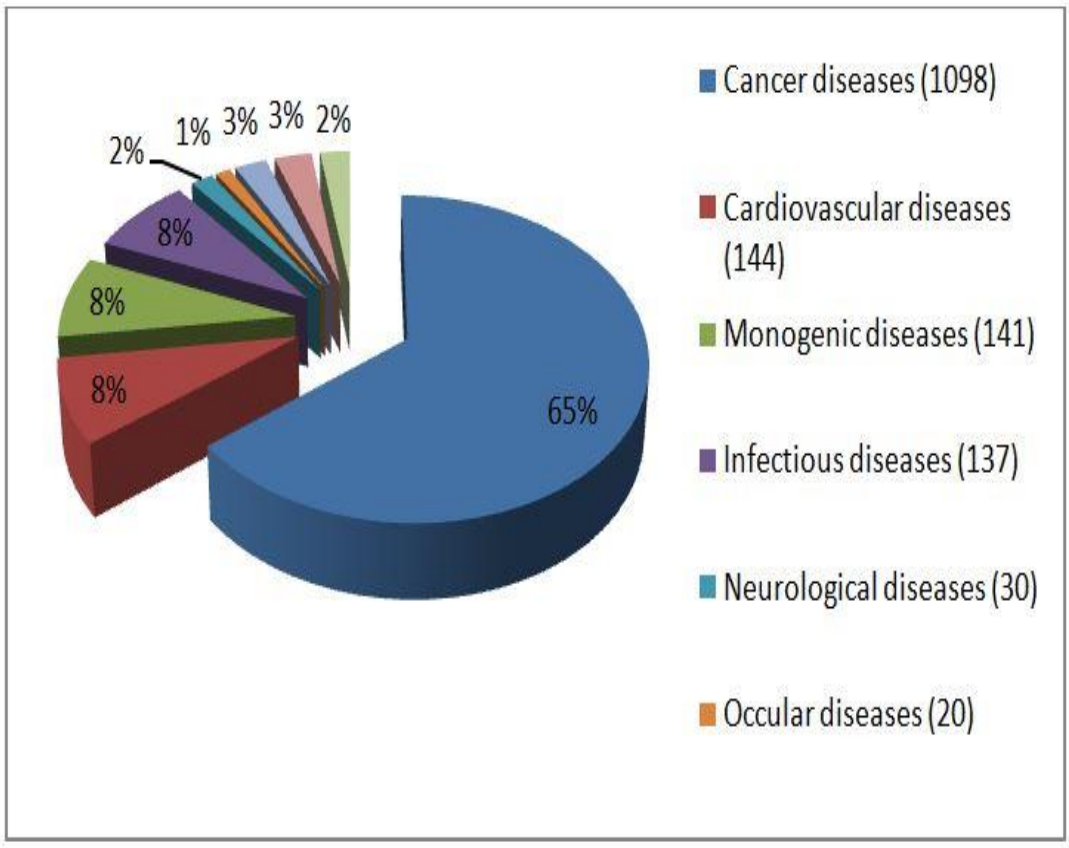

Figure 1.4: This figure illustrates the clinical targets for gene therapy In practice, 65% of gene therapy clinical trials have been directed towards the treatment of cancer [71].

1.4.2 Viral Vector Delivery Systems

Generally speaking, clinical trials have thus far utilised two methods of gene delivery: viral and non-viral. Currently, viral vectors are considered as the most effective of all gene delivery methods for *in-vivo* gene transfer [72-83] As such, it is not surprising to find that they have been the most frequently used vehicles for transferring genes into human cells (Figure 1.5).

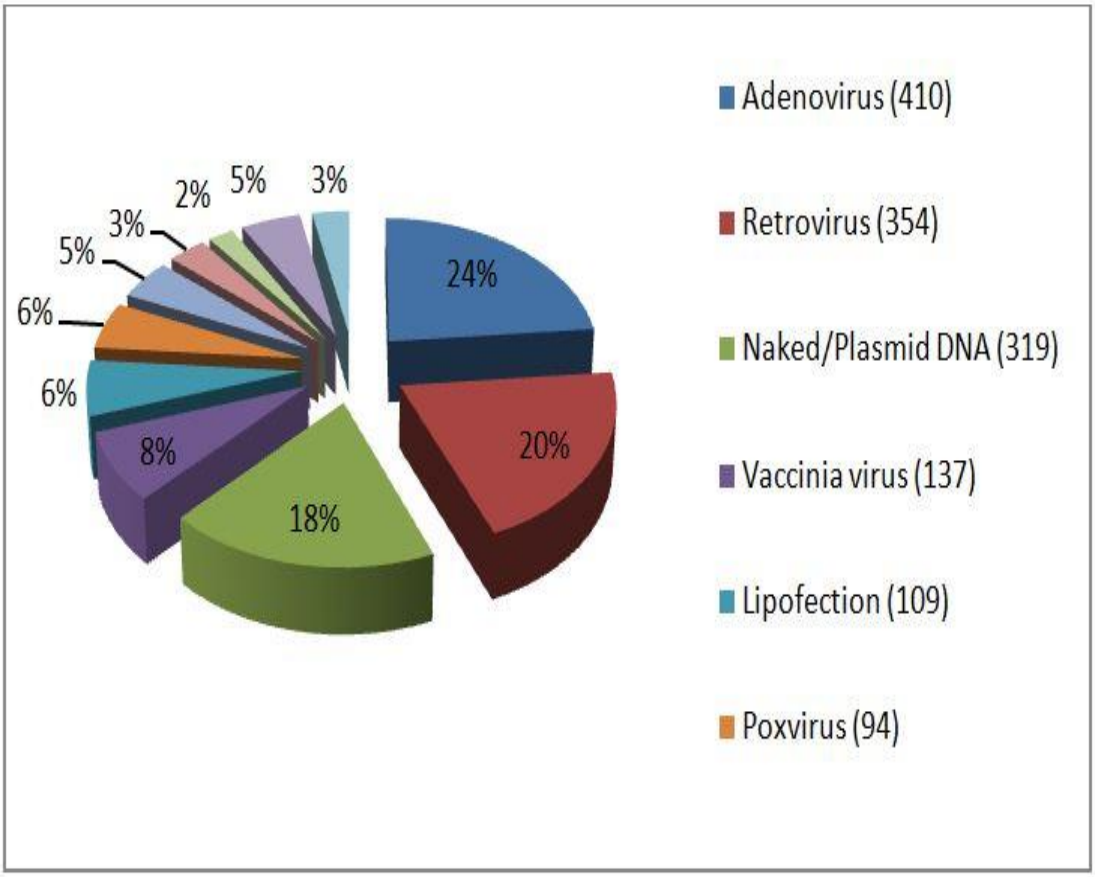

Figure 1.5: Different gene transfer vectors used in clinical settings. By far, adenoviral, retroviral and naked plasmid/DNA have been the most commonly used gene transfer vectors [71].

In most of cases, the viral genome has been genetically modified to make it replication-defective and non-pathogenic. Having established this, it is important to remember that the mammalian immune system is designed to recognise and eradicate non-self proteins. As such, viral gene therapies are always at risk of stimulating the immune system in a way that reduces or even counteracts its therapeutic effectiveness [84-87]. Furthermore, the phenomenon of immune memory results in an enhanced response to previously encountered proteins / antigens, making it difficult for gene therapy to be repeated in patients. Moreover, viruses also present a variety of other potential problems to the patient including toxicity and insertional mutagenesis, as well as posing gene control and targeting issues [88-90]. In addition, there is always the fear that the viral vector, once inside the patient, may recover its pathogenic traits and cause disease.

1.4.2.1 Alphaviruses

Alphaviruses are the major genus of the family *Togaviridae* [91]. Characteristics that make alphaviruses attractive candidates for development of replication-defective viral vectors include:

1. Recombinant alphaviruses induce high-level expression of encoded proteins
2. Following 48-72 hours of protein expression, infected cells die by apoptosis resulting in apoptotic bodies containing high levels of the transgene protein. This could be beneficial for the induction of immune responses via cross-priming [92]
3. Recombinant alphaviruses activate both the innate and adaptive arms of the immune system. Infection of cells results in dsRNA intermediates, known for their immunopotentiating capacity [93]
4. In general, humans do not carry neutralising antibodies against Sindbis virus (SINV) or the Semliki Forest Virus (SFV) that may adversely affect the efficacy of immunization.

Of the alphaviruses, the Semliki Forest virus (SFV) is the best studied [77, 94-99]. In its replication-deficient format, the SFV genome is split into two plasmid vectors. The expression vector contains the non-structural SFV genes (replicon) and the gene of interest, while the helper vector contains the structural proteins. Due to the lack of structural proteins in the SFV replicon, no further virus production occurs, resulting in high safety levels for *in vivo* applications [100].

1.4.3 Non-Viral Vector Systems

While viral vectors have been shown to achieve high rates of transfection, they are also associated with a number of side effects, some of which are potentially lethal. To this end, various non-viral vectors have been designed and developed. Non-viral methods include physical methods such as electroporation, microinjection, gene gun, sonoporation, hydrodynamic gene transfer, cationic lipids and nanoparticles.

Method	Advantages	Disadvantages
Needle injection	Simplicity and safety	Low efficiency
Gene gun	Good efficiency	Tissue damage in some applications
Electroporation	High efficiency	Limited working range; need for surgical procedure
Ultrasound	Potential for site-specific gene delivery	Low efficiency *in vivo*
Cationic lipids	High efficiency *in vitro;* low to medium-high for local and systemic delivery	Limited activity *in vivo*
Lipid/polymer Hybrids	Low to medium-high efficiency in vitro and in vivo; low toxicity	Low activity *in vivo*

Table 1.1: Advantages and Disadvantages of Current Non-Viral Gene Delivery Vector Systems.

Electroporation as a physical delivery system is associated with high transfection efficiency. One of the principal benefits in using electroporation as a vector for gene delivery is that the electropermeabilising current on its own does not alter cellular / tumour growth characteristics, with resultant cell recovery and continued viability. This is an advantage for gene therapy, where therapeutic gene expression requires a duration of normal cellular function.

A further advantage of electro-gene therapy is that the permeabilising pulses limit the distribution of the gene of interest to the pulsed tissue, such that gene expression can be localised and controlled using tissue-specific promoters [101]. When direct injection of naked DNA is followed by the application of electric pulses, enhanced, long lasting transgene expression can be observed relative to gene transfection methods without electroporation [101]. A previously published study by Mir *et al* (1998) reported increased levels of reporter and therapeutic gene expression that persisted for up to 9 months in mouse, rat and monkey models.

Preclinical models have shown the ability of this non-viral vector systems to express sufficient amounts of therapeutically active compounds within tumours, enabling reduced tumour growth or even complete regression. While the transfection efficiency rates of electroporation compare unfavourably with their viral counterparts, electroporation is associated with a much safer therapeutic profile.

1.4.4 Development of a Novel Non-Viral Vector System

In this thesis, we describe the development of a non-viral vector system that has the potential for clinical application in electroporation-based gene therapy. We report a proof of concept Enhanced Expression Vector (pEEV) which encompasses a viral replicase from the Semliki Forest Virus. We aimed to improve upon nuclear entry by the incorporation of a nuclear localization sequence. We demonstrated cytoplasmic expression of the vector. We also investigated the oncolytic capabilities of the pEEV. Furthermore we compared expression levels of the pEEV with standard available plasmids in murine and porcine animal models.

1.5 Cancer as an Immunologic Disease

The concept that the immune system is capable of recognising and eliminating a developing primary tumour in the absence of external therapeutic intervention has existed for nearly a century. There is compelling evidence in both animal and human models to indicate that a functional cancer immunesurveillance process does indeed

exist. Paradoxically, the immune system has the ability to facilitate tumour progression by sculpting the immunogenic phenotype of the tumour, accounting for the development of cancer in immunocompetent individuals.

1.5.1 Immune Tolerance

Shortly after World War II, Sir Frank Macfarlane-Burnet introduced the dichotomy of "the self" and "the other" in an attempt to decipher the science behind immune reactivity. Immune tolerance is the process by which the immune system fails to react to "self" antigens. It occurs in three forms: central tolerance, peripheral tolerance and acquired tolerance.

1.5.1.1 Central Tolerance

This process occurs during lymphocyte development in the thymus and bone marrow. As T- cells mature within the thymus, they develop a recombination of gene segments creating the two chains that make up the T-cell receptor (TCR) for antigen recognition. These TCRs recognise epitopes consisting of short peptide sequences (6-8 amino acids long) derived from body ("self") proteins. These peptide sequences are nestled within a histocompatibility molecule, encoded by the Major Histocompatibility Complex (MHC) as follows:

- MHC Class II for $CD4^+$ T cells
- MHC Class I for $CD8^+$ T cells

1.5.1.2 Peripheral Tolerance

This phase represents immunological tolerance after B and T cells mature and enter the periphery. From a self-reactivity standpoint, these mature B and T lymphocytes are relatively, but not completely, safe. Cells which exhibit self-reactivity are controlled by peripheral tolerance mechanisms.

1.5.1.3 Acquired Tolerance

This phase refers to the immune system's ability to react to external antigens characterized by a specific non-reactivity of the lymphoid tissues to a given antigen which, in other circumstances would likely induce cell-mediated or humoral immunity. One of the most important physiological examples of acquired tolerance occurs during pregnancy, where the foetus and the placenta must be tolerated by the maternal immune system. Acquired tolerance plays a crucial role in the development

of the phenomenon of “oral tolerance”, implicated in the development of inflammatory bowel disease and upper gastrointestinal malignancies.

The concept of “cancer immunoediting” [102-105], emphasises the dual roles of immunity in not only preventing but also shaping neoplastic disease. It envisages that the process of oncogenesis involves three phases: elimination, equilibrium and escape (Figure 1.5) [102-105].

The “elimination” phase represents the original concept of cancer immune surveillance, which, if successful brings about eradication. This phase represents the complete immunoediting process without progression to the subsequent phases. During the “equilibrium” phase, the host immune system and any tumour cell variants that have survived the elimination phase enter into a dynamic equilibrium which is enough to contain, but not eradicate a tumour containing many genetically unstable and mutating tumour cells. The end result of the equilibrium process is a new population of tumour clones with reduced immunogenicity, derived from a heterogeneous parental population and sculpted by the immune system. The “escape” phase refers to a scenario where the tumour cell variants selected in the equilibrium phase can grow in an immunologically intact environment. This breach of the host's immune defences confers tumour cell resistance to immune detection, allowing the tumours to expand and become clinically detectable.

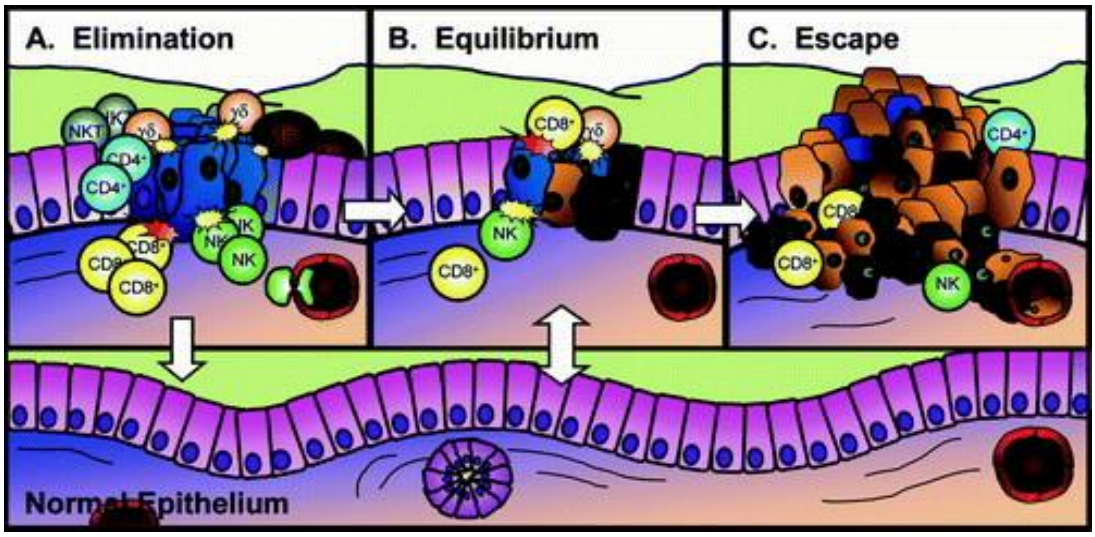

Figure 1.6: The three Es of cancer immunoediting: elimination, equilibrium, and escape. As indicated by the arrows, the immune system may eliminate the tumour in either the elimination or equilibrium phases, returning the tissue to normal [106].

1.5.2 The T Regulatory (Treg) Cell

The existence of a dedicated population of suppressive T cells was the subject of significant controversy among immunologists for many years. However, recent advances in the molecular characterization of this cell population have firmly established their existence and their critical role in the vertebrate immune system [107-114]. Sometimes known as “Suppressor T Cells”, this is a population of specialised T cells that express CD4 and CD25 transmembrane glycoproteins.

Although all cells regulate other cells in some way, Treg cells are exceptional in that their main role seems to be in suppressing the function of other cells [114, 115] (Figure 1.6) . This is achieved in a number of different ways including direct cell-cell contact, mediated by inhibitory molecules like CTLA-4 to cytokine-mediated inhibition via molecules such as IL-10 and TGF-β (Figure 1.7).

While the immunosuppressive potential of Treg cells may be harnessed therapeutically to treat autoimmune disease and to facilitate transplant tolerance, these cells very much represent a ‘double-edged sword’ in that their suppressive function may dampen the immune response, adversely affecting control of both viral and bacterial infections [114]. Crucially, this suppressive function may impede effective immunesurveillance of tumours. Thus, while depletion of Treg cells may disinhibit the immune system allowing for heightened tumour immunesurveillance, leading to tumour rejection, this depletion may concurrently increase the risk of developing autoimmunity [116-121].

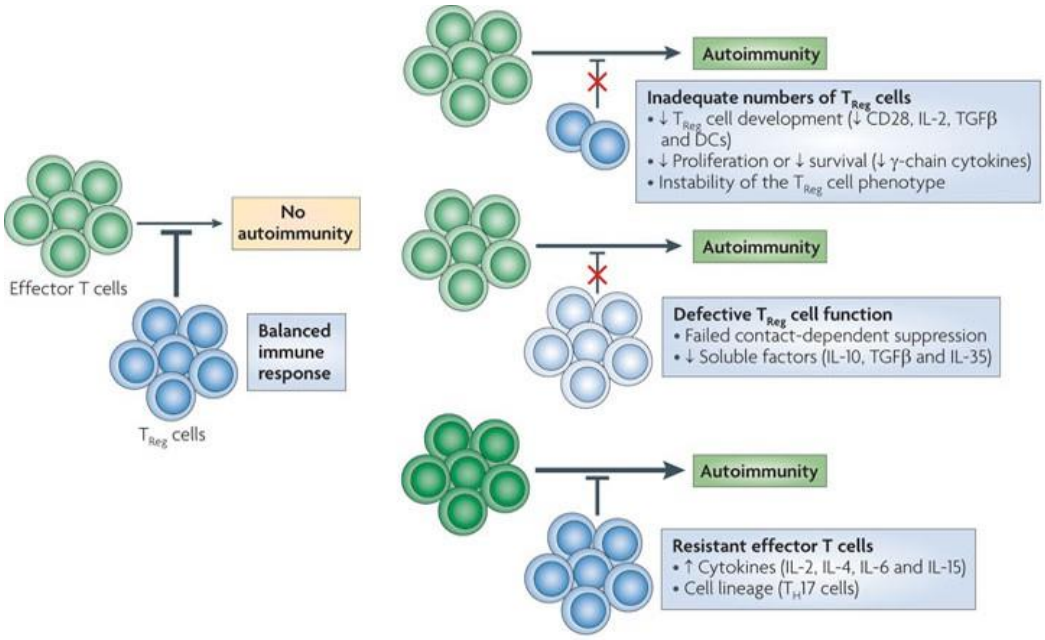

Figure 1.6: A reduction in $CD25^+CD4^+$ Treg cells or attenuation of their suppressive activity may elicit autoimmunity, tumour immunity, microbial immunity and allergy. In contrast, an increase in the number of Treg cells or augmentation of their suppressive activity may establish transplantation tolerance [115].

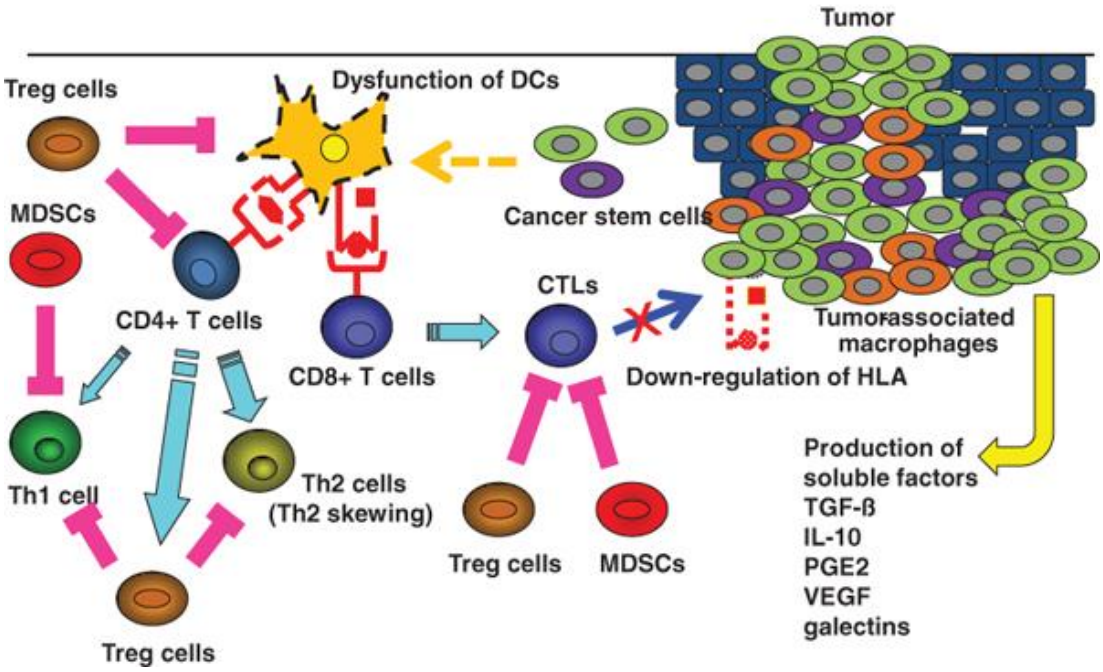

Figure 1.7: Tumours may develop a number of mechanisms to suppress anti-tumour immune responses, such as the dysfunction of DCs, apoptosis of effector T cells, increase of immunosuppressive cells, production of immunosuppressive molecules, down-regulation of HLA expression, and presence of CSCs [122].

1.5.4 Granulocyte Macrophage Colony-Stimulating Factor and the Human B7-1 Co-Stimulatory Molecule

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is an important hematopoietic growth factor and immune modulator. GM-CSF which has profound effects on the functional activities of various circulating leukocytes. It is produced by a variety of cell types including T cells, macrophages, endothelial cells and fibroblasts. Although GM-CSF is produced locally, it can act in a paracrine fashion to recruit circulating neutrophils, monocytes and lymphocytes. Costimulation mediated by costimulatory molecules, such as B7-1, which are ligands for the CD28/cytolytic T lymphocyte associated antigen (CTLA)-4 counter-receptor, plays an important role in the induction of T cell-mediated anti-tumour immunity.

1.5.4 Extension of Clinical Utility

In Chapter 4 of this thesis, we characterised a non-viral vector therapy system; pEEV, with a vastly superior expression capacity when compared to a standard control vector. Having established this, we subsequently validated the use of pEEV as a gene therapy vector carrying murine GMCSF and human b7-1 genes (pEEVGMCSF-B7.1). We used electroporation as a means to facilitate the delivery of the EEV plasmid and assessed the efficacy and immune induction in primary and secondary responses to treatment in murine colon adenocarcinoma and melanoma cancer models.

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Chapter 2

Preclinical evaluation of an endoscopic electroporation system

2.1 Summary

Electrochemotherapy enhances chemotherapeutic drug uptake by delivering electrical pulses causing transient pore formation in the cell membrane that enhance permeabilisation. The EndoVe is an endoscopic device developed to enable the intraluminal delivery of cell membrane permeabilising electroporation pulses. Our preclinical validation studies investigated the efficacy and safety of this non-thermal system in the treatment of gastrointestinal cancer models. Murine gastrointestinal tumour models were used to assess the efficacy of electroporation pulses delivered through the EndoVe in combination with Bleomycin. Tumour cell death, volume and overall survival were recorded. Porcine and canine models were used to assess safety and efficacy. Murine tumour treated with ECT showed excellent responses with cell death induced rapidly via a largely apoptotic type mechanism. Application of the EndoVe to canine gastrointestinal cancer demonstrated successful local endoluminal tumour resolution with no safety or adverse effects noted. The EndoVe offers a non-thermal tumour ablative approach that presents clinicians with a new option for the management of gastrointestinal cancers.

2.2 Introduction

The treatment of gastrointestinal cancers is managed primarily with surgery, in combination with other modalities such as chemotherapy, radiotherapy and small molecule targeted therapies to achieve local disease control and to reduce the risk of recurrence. Improvements in radiological imaging, minimally invasive surgical techniques and a new range of molecular targeted drugs over the last decade have supported a moderate improvement in five year overall survival rates. However, significant challenges remain for patients where the disease has spread, with the US currently reporting survival rates in the case of colorectal cancer at 40% with regional lymph node involvement and at 12.5% where the disease has metastasised.

The effectiveness of many molecular therapeutic agents used in colorectal cancer treatment is also influenced significantly by their ability to access the tumour vasculature, enter into the tumour interstitial cell spaces and cross the cancer cell membrane. Physiological factors such as the presence of a high tumour interstitial pressure can impede to a large degree the ability of therapeutic agents to perfuse the interstitial space while absorption across the membrane is largely non-specific and requires repeated doses that can be toxic to both healthy and tumour tissues [1].

The technology of electroporation offers significant potential as a colorectal neo-adjuvant therapy in that it can facilitate both perfusion and absorption of low dose chemotherapy agents. Electroporation is a term used to describe the permeabilisation of the cell membrane following the application of a short and intense electric pulse. The permeabilisation can be temporary (reversible electroporation) or permanent (irreversible electroporation) as a function of the electrical field magnitude and duration, and the number of pulses [2]. It facilitates targeted chemotherapy absorption, referred to as electrochemotherapy (ECT), and has been increasingly employed clinically over the last 10 years. Positive outcomes have been published from the clinical experience in terms of tumour reduction, with an overall objective response rate of between 80-90% [3-7]with associated quality of life improvement.

We have developed the EndoVe device which enables the endoscopic electroporation treatment option of gastrointestinal cancers. The EndoVe initially applies a vacuum directly to the tumour tissue resulting in a reduction of the tumour interstitial pressure, thus facilitating chemotherapeutic distribution within the

interstitial space. The vacuum also brings the tumour into direct contact with the EndoVe electrodes which deliver a set of 100μsec electrical pulses to the underlying tissue. The electroporation pulse results in the tumour becoming extremely porous enabling chemotherapy drugs such as Cisplatin or Bleomycin to be absorbed up to a 1000-fold more effectively [2, 8-10]. The benefit of this approach is that significantly less chemotherapy is required for a substantially more effective anti-tumour response and the patient benefits from a reduction in the tumour without the side effects due to the lower drug dose used. Surrounding healthy tissue structures remain preserved after the procedure allowing wider margins around the tumour to be rapidly treated.

The aim of the investigation was a pre-clinical evaluation of the EndoVe prior to a phase I/II clinical study. We sought to validate the efficacy of the system in the treatment of cutaneous murine gastrointestinal tumours cell lines and to assess the safety and utility of endoscopically delivered electroporation through evaluation in a porcine model and by treating canine colorectal tumours.

2.3 Materials and Methods

2.3.1 Cell Tissue Culture

Established colorectal cell lines CT26, CMT93 (Mouse), HT29, LoVo and CACO-2 (human) were obtained from the American Type Cell Collection (Manassas, VA). CT26 and CMT93 cell lines were maintained in DMEM, CACO-2 cell line was maintained in EMEM, LoVo cell line maintained in F12:HAMS media, HT29 cell line maintained in McCoy's 5A, all supplemented with 1% penicillin/streptomycin, 10% (v/v) foetal calf serum and 300µg/ml L-glutamine at 37°C, 5% CO_2.

2.3.2 Animals

2.3.2.1 Ethics Statement

All animal husbandry and experimental procedures were approved by the University College Cork Animal Experimentation Ethics Committee and carried out under licenses issued by the Department of Health, Ireland as directed by the Cruelty to Animals Act Ireland and EU Statutory Instructions.

2.3.2.2 Mice

Female BALB/c, C57BL/6J and MF1-nu/nu mice (6-8 weeks) were obtained from Harlan Laboratories (Oxfordshire, England).

2.3.2.3 Pigs

Pigs (Landrace; n=20) were obtained from Teagasc, Moorepark, Ireland. During procedure pigs were anaesthetised by inhalation of 1.5-2 % Isoflurane delivered via oxygen as a carrier gas at a flow rate of 4-6 litres/minute.

2.3.2.4 Dogs

All dogs had naturally occurring intraluminal tumours confirmed through obtaining endoscopic biopsies endoscopically and subsequent histopathologic examination. Dogs were premedicated with a combination of acepromazine (PromAce, Fort Dodge Animal Health, Iowa, USA; 0.02 mg/kg) and Buprenorphine. General anesthesia was induced using Propofol (4mg/kg) and maintained with Isoflurane (oxygen flow rate 100 mg/kg). All animals received Carprofen (2-4 mg/kg) for pain relief.

2.3.3 Electric Field Modelling

Electrical field modelling was carried out using FEM modelling. The electric field distribution was calculated without taking into account changes in conductivity due to electroporation – it represents the static electric field distribution.Voltage used: 1000 V (FemLab).

2.3.4 Tumour Induction and Growth Assessment

For routine tumour induction, 1 x 10^6 CT26 or 5 x 10^5 CMT93 tumour cells suspended in 200 µl of serum free DMEM were injected subcutaneously into the flank of the female BALB/c or C57BL/6J. Tumour volume was calculated according to the formula $V=ab^2\pi/6$, where a is the longest diameter of the tumour and b is the longest diameter perpendicular to a. Tumour growth curves were constructed using volumes calculated in this manner. Mice were euthanized when the tumour diameter exceeded 1.7cm^3. Survival was recorded and presented as Kaplan-Meier survival curves.

2.3.5 Drug Delivery

The dose of Bleomycin (Mayne Pharma (Ireland) Ltd.) was dependent on route of administration. In murine electrochemotherapy studies, Bleomycin was administered intra-tumourally at a dose of 1000 IU/ $cm^{3.}$ In cases of canine electrochemotherapy, Bleomycin was administered intravenous (i.v.) as a bolus over 30 seconds at a dose of 15000 IU/m^2 of body surface area.

2.3.6 Electroporation Parameters

Square wave electroporation pulses were generated with an ePORE Gx (Cork Cancer Research Centre, Cork, Ireland) square wave electroporation generator, and delivered to tissue using the EndoVe device (Cork Cancer Research Centre, Ireland). Eight square-wave pulses were delivered sequentially, at a frequency of 1Hz, for duration of 100µs each.

2.3.7 Assessment of EndoVe-delivered Electrochemotherapy

Efficacy of EndoVe-delivered ECT was ascertained by treating subcutaneous murine tumours. Prior to electroporation a surgical incision was made to expose the tumour

directly to the electrodes with the same steps followed for the control groups. Following electroporation, the skin was closed with absorbable Vicryl sutures.

2.3.8 Histopathology

2.3.8.1 Haematoxylin and Eosin Staining

Tissue samples were fixed in 10 % v/v buffered formalin and embedded in paraffin. Serial sections were cut at 5 µm and mounted on slides. Standard H&E staining was performed on murine, canine and porcine tissue samples.

2.3.8.2 Apoptosis Staining

Evaluation of the tumours for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL; Roche) staining was utilised as described previously [130].

2.3.9 Statistical Analysis

Experimental results were plotted and analyzed for significance with Prism 4 software (GraphPad software Inc, CA, USA). $P < 0.05$ was considered significant.

2.4 Results

2.4.1 Design and Electrical Field Modelling on the EndoVe

The EndoVe was designed to incorporate two plate electrodes which were positioned parallel apart within its chamber (Figure 2.1). Electrical field modelling of various options demonstrated that the strongest, most efficient and homogenous electrical field was generated where the plate electrodes formed a lip around the outside edges of the chamber, enabling tissue underneath the chamber to also be electroporated. With the plates positioned 1.1cm apart, 1.0cm in depth, 2.5cm in length and with an applied voltage of 1000V/cm, a 100μsec pulse was able to fully electroporate tissue within the chamber and underneath the device to a depth of several mm's (2-5mm at 1000 V/cm). Attempts to engineer a prototype with a depth of greater than 1.0cm resulted in reduced manoeuvrability and the prototype catching on the epiglottis. In clinical practise, the device was placed multiple times across tumour tissue to ensure full tumour tissue coverage with the total volume of tissue treatable with a single pulse calculated between 2.75 and 3.75 cm^3.

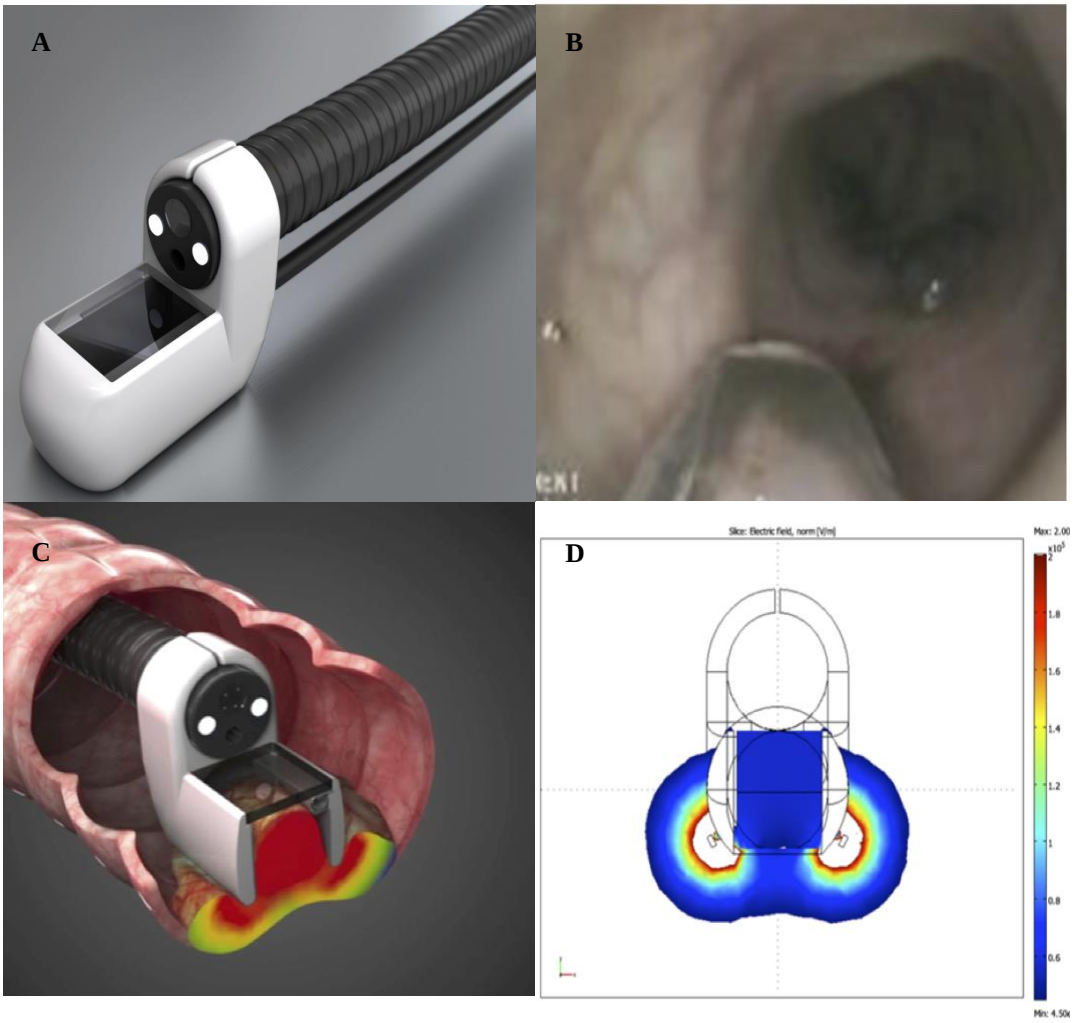

Figure 2.1: The EndoVe. **A:** EndoVe attaches to conventional endoscopy. **B:** EndoVe ECT in a porcine model **C:** Schematic representation of EndoVe ECT and energy applied. **D:** Electrical field modelling of EndoVe at 1000V/cm.

2.4.2 Effect of EndoVe Electrochemotherapy in Murine Gastrointestinal Tumour Models

We evaluated the efficacy of the EndoVe as a novel ECT delivery probe by treating subcutaneously established CT26 and CMT93 xenografts (Figure 2.2). The outermost layer of the skin, the stratum corneum, is a resistive/non-conductive obstacle to successful electroporation of subcutaneous tumours, as it acts to dissipate delivered pulses [11]. For intraluminal tissue, this is not a barrier to treatment with electroporation. To assess the effect of intraluminal EndoVe-delivered electrochemotherapy, subcutaneously induced xenografts were injected intra-tumourally with Bleomycin and then electroporated using the EndoVe. Tumours that received electrochemotherapy displayed significant tumour volume reduction compared with controls (Figure 2.2A and B). All ten tumours in the CT26 treated group (9/10 CMT93) responded in this manner with tumour reduction observable within 48hrs of the procedure (Figure 2.2C and D). Histological examination using H&E staining of tissue sections from ECT-treated tumours removed at necropsy 48 hours after treatment showed extensive denucleation of the treated tumour tissue (Figure 2.3A i and ii), in contrast to the control group. H&E staining was also used to assess the impact of ECT on porcine healthy rectal and oesophageal tissues (Figure 2.3A iii-vi) 48 hours post treatment. Results demonstrated similar denucleation evidence as in the murine studies although not to the same extent along with some interstitial inflammation. Overall preservation of tissue architecture was noted along with an absence of any significant necrosis or haemorrhage. TUNEL staining for apoptosis in the murine ECT treated tumours was overwhelmingly positive (Figure 2.3B) and across a range of gastrointestinal tumour models tested, EndoVe ECT with Bleomycin was able to induce apoptosis in <6 hours post treatment (Table 2.1).

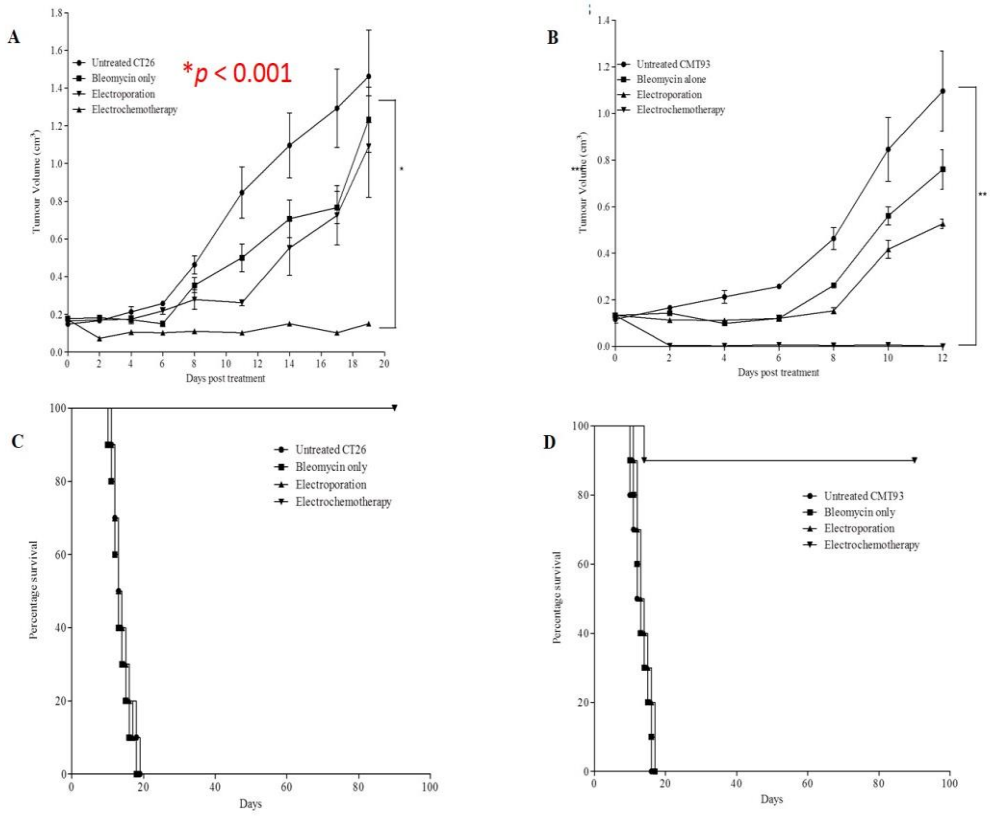

Figure 2.2: Therapeutic effect on established solid tumours. **A**: CT26 tumour growth curve. Each Balb/C mouse subcutaneously injected with 1 x10^6 CT26 cells in the flank. When tumours reached an approximate size of 100mm^3 they were treated with Bleomycin (■), electroporation (▲), electrochemotherapy (▼) or untreated (●).Tumour volume was calculated using the formula $V=ab^2/6$. Mice were sacrificed when tumour reached the ethical accepted tumour volume. 19 days post treatment electrochemotherapy significantly delayed tumour growth compared to untreated CT26 tumour (***p<0.001) **B:** CMT93 tumour growth curve. Each C57BL/6J mouse subcutaneously injected with 5 x10^5 CMT93 cells in the flank. When tumours reached an approximate size of 100mm^3 they were treated with Bleomycin (■), electroporation (▲), electrochemotherapy (▼) or untreated (●).12 days post treatment electrochemotherapy significantly delayed CT26 tumour growth compared to untreated tumour (**p<0.01). **C:** Representative Kaplan-Meier survival curve of CT26 tumour showing response to each treatment. Survival curve shows 100% survival up to 150 days post treatment with all other groups sacrificed by day 21. Similar results were obtained in two independent experiments. **D:** Representative Kaplan-Meier survival curve of CMT93 tumour showing response to each treatment. Survival curve shows 90% survival up to 150 days post treatment with all other groups sacrificed by day 19. Similar results were obtained in two independent experiments.

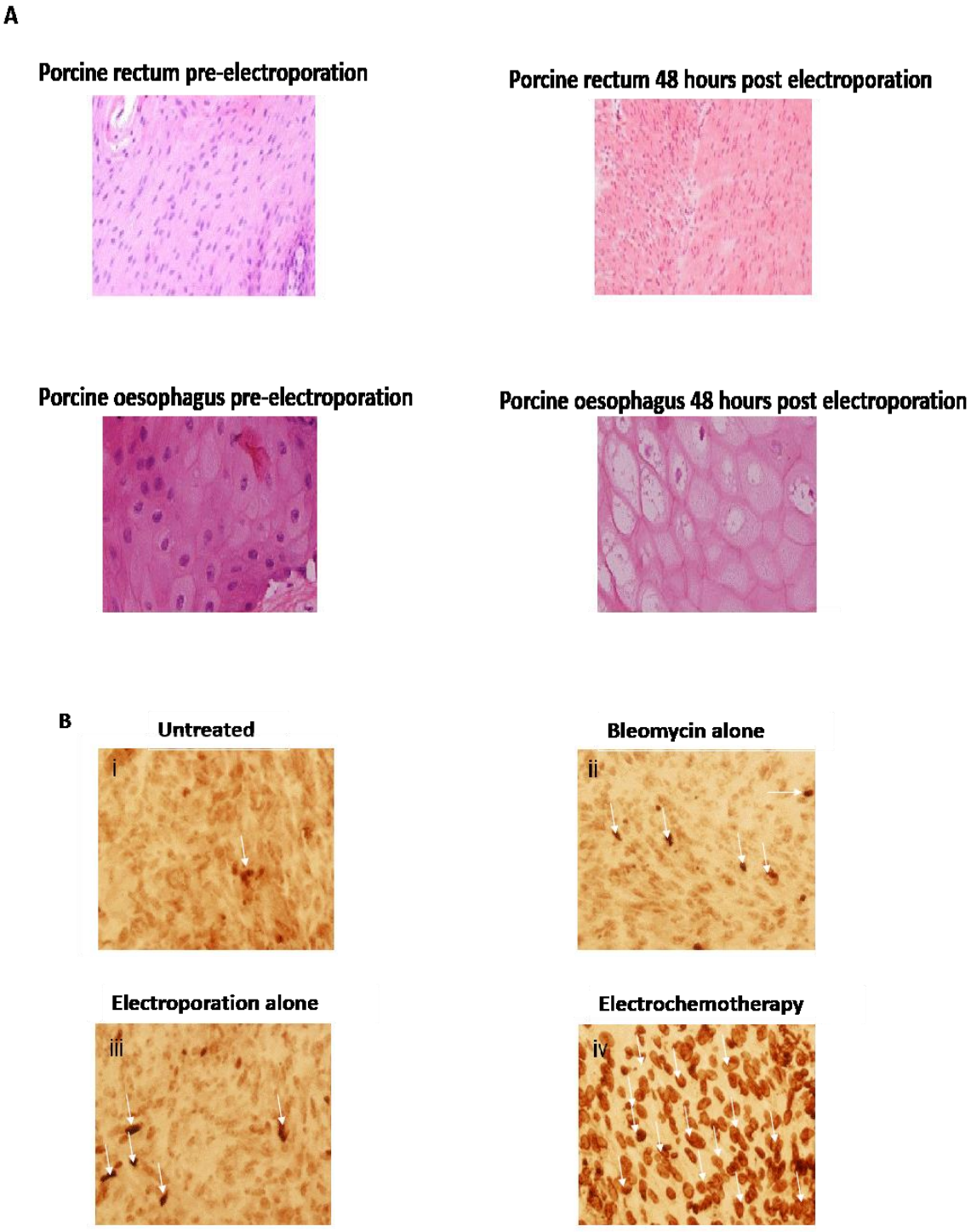

Figure 2.3: (**A)** H&E staining of CT26 tumours and porcine tissue. **A: i:** Murine CT26 untreated tumour. **ii:** Murine CT26 48 hrs post ECT. **iii:** Porcine healthy rectum. **iv:** Porcine healthy rectum 48 hrs post ECT. **v.** Porcine healthy ooesophageal. **vi.** Porcine healthy ooesophageal 48 hrs post ECT; (**B)** Apoptotic death evaluated using TUNEL staining. Tumours were removed from mice 48 hours post treatment; Paraffin embedding and stained sections were examined microscopically. Samples shown represent **i:** untreated CT26, **ii:** Bleomycin alone**, iii:** electroporation alone and **iv:** ECT treated CT26 tumours. Apoptosis is indicated by the cell nucleus staining dark brown as is seen in the ECT treated CT26 tumour tissue.

	CT26	CMT93	HT-9	CACO2	LoVo	OE19
Untreated	3%	4%	4.30%	3%	2.80%	4%
EP alone	5.60%	7.50%	8.10%	8.50%	6.10%	7.70%
Bleo alone	4.30%	5.40%	4.90%	6%	3.60%	5.50%
ECT 3hr	88.30%	89%	94%	99.50%	73.70%	80%
ECT 6hr	97.30%	96.80%	97%	99%	97.90%	97.20%

Tunel activity of the tumour cell lines at various timepoints after treatment

Table 2.1: Percentage of cells *in vitro* activity showing TUNEL activity as measured on the FACS.

2.4.3 Safety of EndoVe Application: Porcine Assessment

The EndoVe was applied to intraluminal pig tissues to validate the efficacy and safety of the system. Tissues tested included the oesophagus and rectum/colon. Due to the absence of a suitable porcine gastrointestinal tumour model we tested the EndoVe device in twenty pigs to a) safely under vacuum pressure draw tissue into the chamber with increasing negative pressure from -100 to -800mmHg and b) treat tissue at a 1Hz frequency, eight 100μsec pulses at a voltage of either 1000V/cm or 1400V/cm and subsequently biopsied for H&E staining 48hrs post procedure (Figure 2.3B). All 20 porcine cases were performed with no trauma, burns or perforations observed in the seven days post procedure follow up.

2.4.4 EndoVe-delivered Electrochemotherapy of Spontaneous Canine Colorectal Tumours

Two dogs with spontaneous, inoperable colorectal tumours were treated with EndoVe-delivered ECT. Both animals received intravenous (i.v.) Bleomycin and were treated under a short general anaesthesia.

2.4.4.1 Case 1

A 10 year old sheltie with rectal bleeding, diarrhoea and loss of condition, including significant recent weight loss was presented. Colonoscopy revealed an obstructing colorectal mass about 10 cm proximal from the anal verge (Figure 2.4A). Histopathologic analysis confirmed that this lesion was an adenocarcinoma. The tumour was deemed unsuitable for surgical resection and the dog was referred for ECT. The tumour was treated with two separate sessions of ECT on days 15 and 34. After each ECT session, the size of the mass was visibly decreased (Figure 2.4B and C). Complete regression was obtained after two ECT sessions (Figure 2.4D). No relapse was observed over two and a half years following the last treatment. Complete regression was confirmed by biopsies confirming the presence of no residual tumour tissue (Figure 2.5).

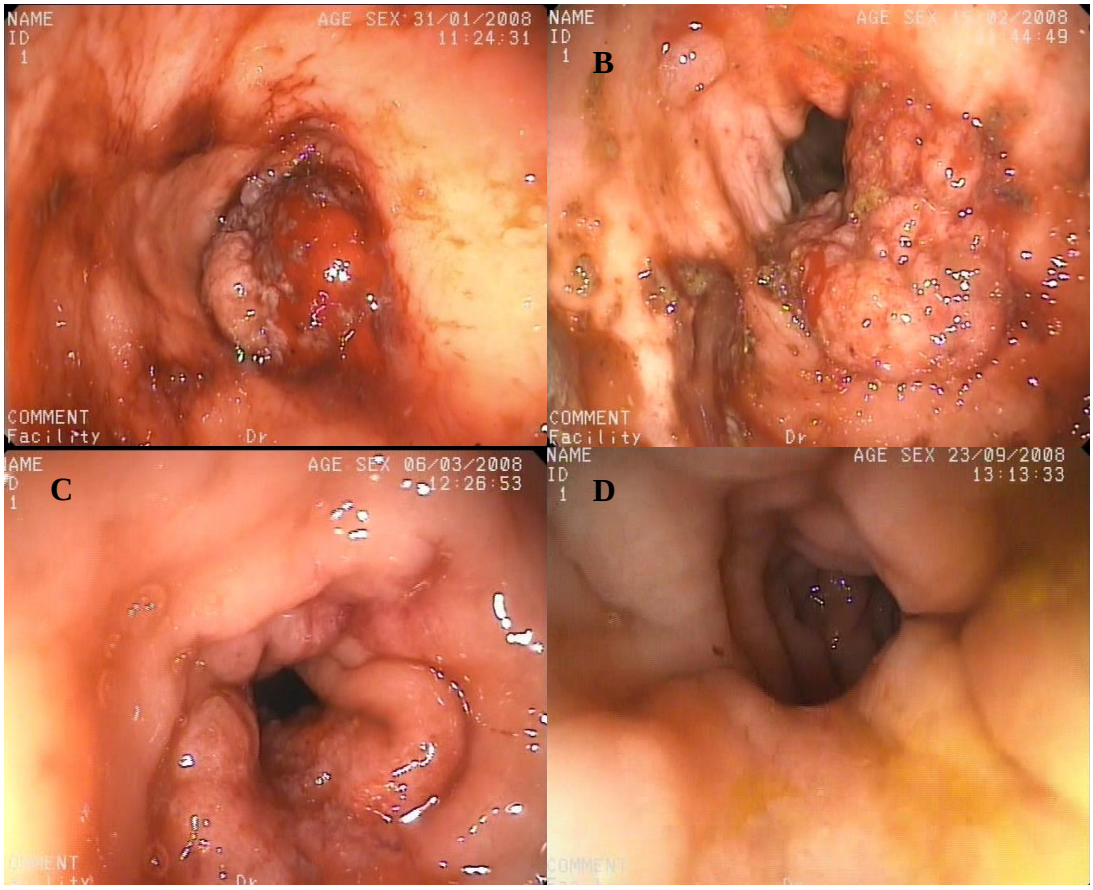

Figure 2.4: ECT of a canine rectal adenocarcinoma. **(A)** Endoscopic images of an obstructing tumour 10cm from the anal verge, (**B**) 15 days following the first session of ECT; **(C)** continuing tumour regression after a second session of ECT; **(D)** complete tumour regression following 2 sessions of ECT.

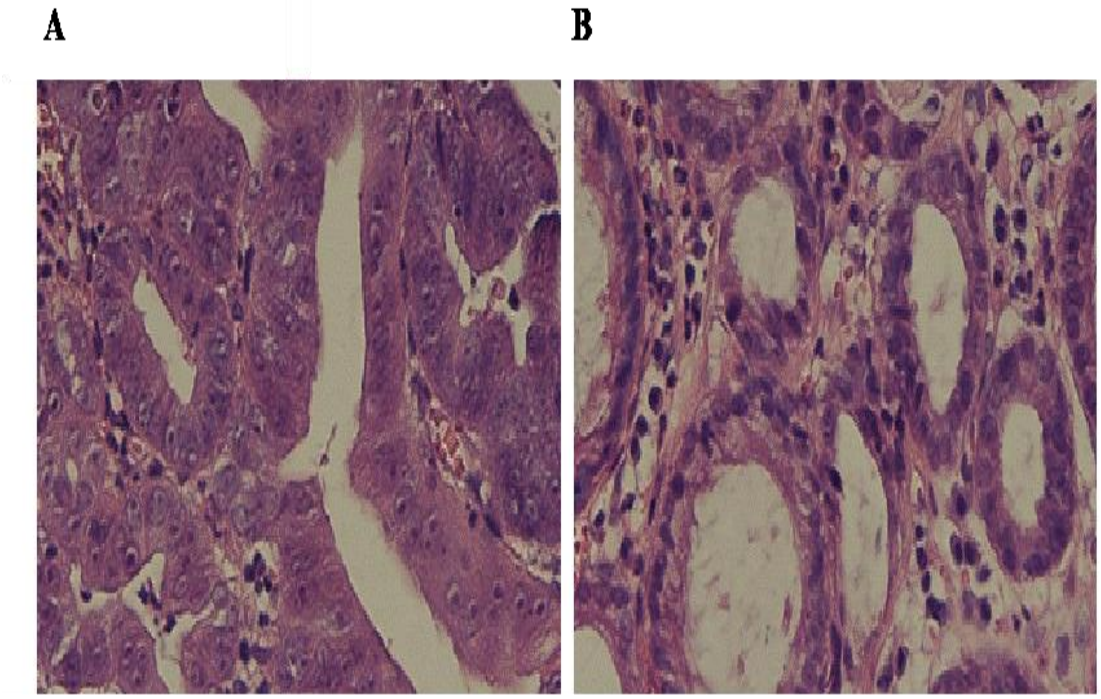

Figure 2.5: Haemotoxylin and Eosin staining demonstrating **(A)** canine colorectal adenocarcinoma pre-electrochemotherapy and **(B)** complete histologic regression 2 years following electrochemotherapy

2.4.4.2 Case 2

A five year old terrier presented with diarrhoea, weight loss and lethargy. Colonoscopy revealed a partial obstructing colonic mass of 6 cm in length from the anal verge (Figure 2.5A). Histopathologic analysis confirmed that this mass was a lymphoma. The tumour was referred for ECT. Complete regression was obtained after two ECT sessions (Figure 2.5 B and C). No relapse was observed two years post treatment (Figure 2.5D). Tumour regression was also confirmed histologically (Figure 2.6).

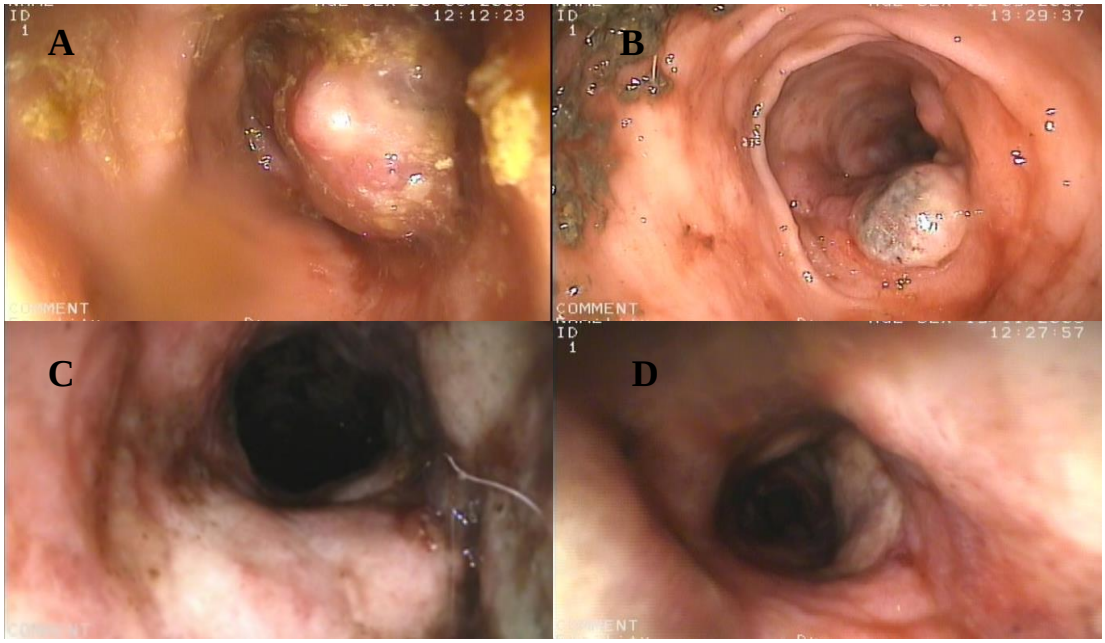

Figure 2.5: ECT of a canine lymphoma. (**A**) Endoscopic images of a rectal tumour 6cm from the anal verge, (**B**) 23 days after treatment with ECT, (**C**) 46 days after treatment with ECT, (D) 2 years following treatment.

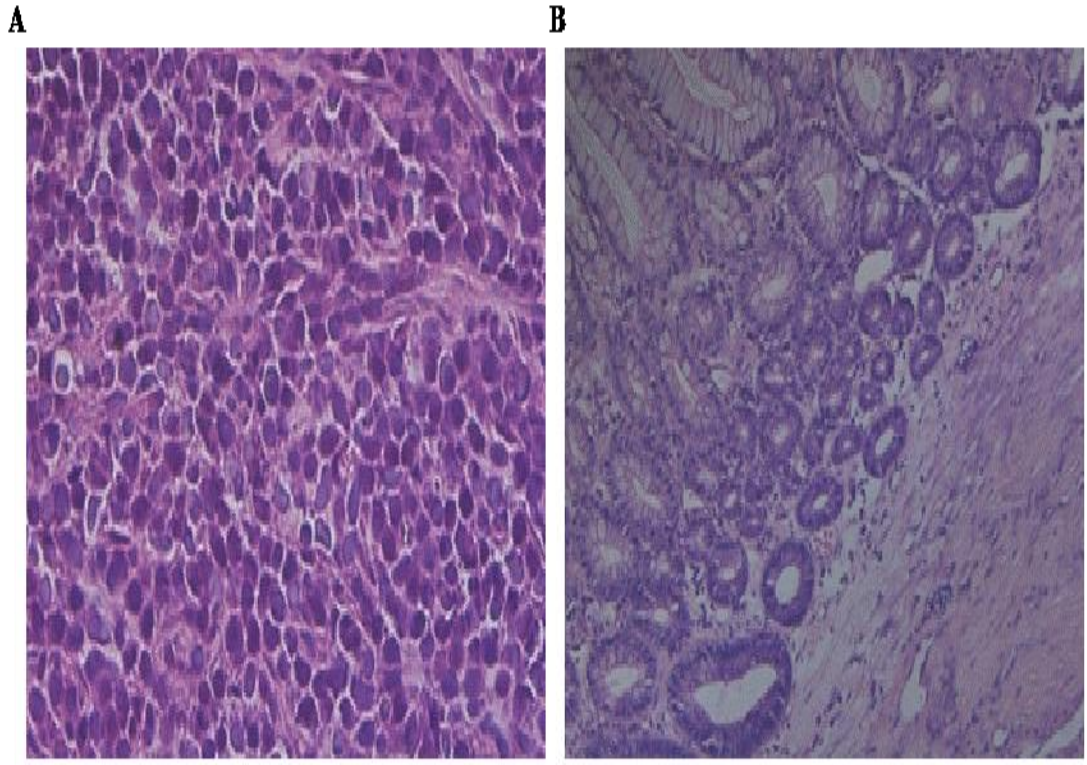

Figure 2.6: Haemotoxylin and Eosin staining demonstrating **(A)** canine colorectal lymphoma pre-electrochemotherapy and **(B)** complete histologic regression 2 years following electrochemotherapy

2.5 Discussion

Minimally invasive tumour ablation is a valuable clinical option in the cancer therapeutic armamentarium with electroporation presenting itself as a method with exciting potential clinical applications. The effectiveness of both reversible and irreversible electroporation in tumour ablation clinically has been reported by multiple investigators [3, 4, 12-14]. Clinical experience with reversible electroporation has been published extensively as a therapy that improves tissue permeability to specific chemotherapeutic agents providing local control of cutaneous cancers unsuitable for surgery and resistant to chemo/radiotherapy [15-18]. Surrounding healthy tissue structures remain preserved after the procedure allowing wider margins around the tumour to be rapidly treated. Published experiences with ECT demonstrate that the non-thermal apoptotic type cytotoxic effects are confined to the region of tumour tissue covered by the electrical field. Because the dose of chemotherapy used for a single therapy, whether administered intralesionally or systemically, is usually less than one twentieth of the cumulative dose used in a standard systemic regimen, there are minimal systemic side effects [17-19].

To date, the equipment for electroporation has not existed to allow for endoscopic delivery to gastrointestinal tissues. We have demonstrated that electroporation delivered with the EndoVe device in combination with Bleomycin can offer a potent targeted anticancer modality for gastrointestinal tumours while also generating a favourable immunological modulation occurring in the period post treatment.

The EndoVe device (Figure 2.1Aand B) works in conjunction with routine endoscopic equipment where the device attaches over the distal end of the endoscope, without obscuring the lens. As such the device allows for both optimal manoeuvrability of the electrode system to the targeted tissue and for contact between the electrode system and the targeted tissue to be achieved. Incorporation of an external lip into the shape of two plate electrode contained within the device chamber allowed for the electrical field generated to permeate the tissue under the device not drawn into chamber under vacuum (Figure 2.1C). The transparent roof of the device chamber allows for tissue capture, and its subsequent release, to be visually confirmed (Figure 2.1B). Initial prototypes of the device have been made

from rigid materials making it more suitable for colorectal application. A flexible version is currently under development for oesophageal and gastric clinical applications.

Employing previously optimized electroporation and chemotherapy parameters [8, 17] murine colorectal tumours were treated using electroporation delivered via a miniature EndoVe. Mice in the untreated, electroporation only and Bleomycin only groups were culled by day 12, whereas the combination treated tumours all showed excellent regression (CT26 10/10; CMT93 9/10) without recurrences (100 days). Cell death was rapid with TUNEL staining and FACS analysis on several gastrointestinal cell lines indicating over 90% of the cells undergoing apoptosis within six hours of treatment (Figure 2.3B and Table 2.1).

We assessed the EndoVe device for safety and operation in a twenty pig study where the device was applied to ooesophageal, gastric and colorectal tissues. Tolerances for the vacuum (-50mmHg to -800mmHg) were assessed without obvious indications of trauma post procedure while simultaneously electroporation pulses were also deployed to the tissue over a voltage range of 500-1400V/cm. Technically electropermeabilisation occurs above 500V/cm and in our experience in the range up to 1200V/cm the procedure was completed smoothly, above which involuntary muscular contractions become an issue. The current delivered was in the range 8-12A per 100 μsec pulse. Our calculations found that the total energy deployed to the tissue was approximately 25-60 joules per pulse and we demonstrated that the delivery of the electroporation pulse resulted in a change of less than 1°C.

Limitations in the design of the current device were encountered which related to its manoeuvrability, specifically difficulties were encountered in the porcine and canine models relating to manipulating the rigid device around the epiglottis which presented a challenge for the endoscopist. A revised design which is made of flexible material and shallower in depth should help overcome these difficulties. Placement and attachment onto a target pre-stained with india ink was achieved, with tissue traction easily achieved through the applied vacuum prior to delivery of the electroporation pulses.

In the canine tumours treated the device was repositioned after each delivered pulse to ensure all of the tumour surface area received a minimum of one

application. The endoscopist worked from a mental map of a Venn diagram approach to ensure all of the target area was treated. Not all tumour tissue treated was amenable to being drawn into the EndoVe chamber although placement directly onto the surface alone was sufficient to 'porate' the underlying tumour. The procedures required an assistant to control the vacuum and electroporation generator while the endoscopist manipulated the device into position. Each step of placement, vacuum and delivery of pulse taking between 30-60 seconds, with the entire procedure completed in 10-20 minutes. No adverse events were noted, or signs of perforation in the treated tissues in any of the porcine and canine procedures, with complete tumour resolution observed in the two obstructive canine cases presented (Figures 2.4 and 2.5).

Clinical application of endoscopic electroporation which is a non-thermal, targeted outpatient technology offers clinicians a new option for the treatment of gastrointestinal cancers. Electroporation may be clinically considered initially for the palliation of symptoms with local control of the tumour.

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Chapter 3

Enhancement of Electroporation-Facilitated Immunogene Therapy via Treg Depletion

3.1 Summary

Regulatory T cells (Treg) can negatively impact tumour antigen-specific immune responses after infiltration into tumour tissue. However, depletion of Treg can facilitate enhanced anti-tumour responses, thus augmenting the potential for immunotherapies. Here we focus on treating a highly aggressive form of cancer using a murine melanoma model with a poor prognosis. We utilise a combination of Treg depletion and immunotherapy plasmid DNA delivered into the B16F10 melanoma tumour model via electroporation. Plasmids encoding murine GM-CSF and human B71 were transfected with electroporation into the tumour and transient elimination of Treg was achieved with CD25 depleting antibodies (PC61). The combinational treatment effectively depleted Treg cells compared to the untreated tumour and significantly reduced lung metastases. The combination treatment was not effective in increasing the survival, but only effective in suppression of metastases. These results indicate the potential for combining Treg depletion with immunotherapy based gene electrotransfer to decrease systemic metastasis and potentially enhance survival.

3.2 Introduction

Although malignant melanoma comprises less than 5% of malignant skin tumours; it is responsible for almost 60% of lethal skin neoplasms [1, 2]. The incidence of malignant melanoma continues to rise, while at the same time current treatment regimes show limited impact on survival [1, 2]. Existing treatment strategies include surgery, chemotherapy and radiotherapy, however many patients succumb to local regional and distal recurrences [3-5]. Current management of melanoma relies on primary prevention and early detection of disease [3, 6] and new treatment approaches that are more tolerable, reduce the risk of relapse and do not impinge on the patient's quality of life are urgently required.

Conventional therapies have demonstrated poor anti-cancer effects for reasons such as chemo-resistance and rapid metastasis [7-10]. Due to its inherent poor prognosis compounded by ineffective treatment regimens, there is a significant drive to design diverse treatment strategies against melanoma, with immunotherapy representing a key therapy focus [11].

There are a number of reasons to support the theory that malignant melanoma is an immunogenic tumour. Firstly, melanoma tumours are typically infiltrated with lymphocytes and a lymphocyte-depleted tumour is typically associated with a poorer prognosis [12, 13]. Malignant melanoma is also more common in immune suppressed, or immune compromised patients [14]. Additionally, a small proportion of melanoma tumours undergo spontaneous regression which is postulated to be related to immune surveillance [15].

The goal of immunotherapy is to increase overall anti-tumour immunity and thus represents a potent means for cancer treatment. However, a major obstacle to the success of immunotherapy is the presence of negative factors that inhibit the immune system such as regulatory T cells [16]. Regulatory T cells (Treg) have been implicated as one of the major suppressive mechanisms of anti-tumour immune responses. Increased levels of Treg in tumours are often associated with poor clinical outcome and tumour progression in various tumour entities [16]. In healthy immune homeostasis, Treg (a subset of T cells) play a crucial role in maintaining immunological unresponsiveness to self-antigens and in suppressing excessive immune responses that would otherwise be harmful to the host [17]. However, in the tumour environment, Treg-induced immune suppression poses a significant barrier to

anti-cancer responses targeted by immune-therapeutic strategies [17-19]. We, and other groups, have demonstrated that an increase or decrease in Treg cells has a direct influence on the effect of an immunotherapy approach [20]. Other studies have used different therapeutic approaches including IL-12 and IL-2 which has had significant therapeutic responses [21-23]. We have previously reported that an immunotherapy DNA plasmid encoding the cytokine GM-CSF (pGMCSF-B7.1), combined with pre-conditioned Treg depletion, was effective in the treatment of murine fibrosarcoma [24].

3.3 Study Aims

To determine the efficacy of immunotherapy in aggressive malignant melanoma we electrically delivered a plasmid encoding GM-CSF, in combination with Treg depleting anti-CD25 to mice receiving the melanoma tumour cell line B16F10. Our results demonstrate that this combinational approach significantly reduced tumour volume and systemic lung metastases and significantly improved overall survival time and thus represents a promising therapeutic approach against melanoma.

3.4 Materials and Methods

3.4.1 Plasmids

The mammalian expression vector pMG was purchased from Invivogen (Cayla SAS, Toulouse, France). A version of this plasmid, designated pGT141, containing the murine GM-CSF and human B71 genes transcriptionally controlled from the hEF1-HTLV and CMV promoters respectively was designed and cloning was performed on contract by Invivogen. Human B7-1 cDNA has been shown previously to function in a murine setting. The inserts were confirmed by sequencing. Plasmids were propagated in *E coli* strain Top10 and purified on endotoxin-free Qiagen-tip 500 columns (Qiagen).

3.4.2 Animals and tumour induction

3.4.2.1 Ethics Statement

All animal husbandry and experimental procedures were approved by the University College Cork Animal Experimentation Ethics Committee and carried out under licenses issued by the Department of Health, Ireland as directed by the Cruelty to Animals Act Ireland and EU Statutory Instructions.

3.4.2.2 Mice

Female C57BL/6 (6-8 weeks) mice were obtained from Harlan Laboratories (Oxfordshire, England). All *in vivo* experiments were approved by the ethics committee of University College Cork and carried out under licenses issued by the Department of Health, Ireland as directed by the Cruelty to Animals Act Ireland and EU Statutory Instructions. Mice were kept at a constant room temperature (22^{o}C) with a natural day/night light cycle in a conventional animal colony. Standard laboratory food and water were provided *ad libitum*. The B16F10 cell line was obtained from the ATCC and cultured in DMEM supplemented with 10% v/v foetal calf serum, 300µg/ml L-glutamine. Tumour cells from a mid-log phase culture were harvested by brief exposure to 0.05% trypsin/EDTA solution, washed twice with PBS. For routine tumour induction, 5 x 10^5 tumour cells suspended in 200 µl of serum free DMEM were injected subcutaneously into the flank of mice. Following tumour establishment, tumours were allowed to grow and develop and were monitored by alternate day measurements in two dimensions using Vernier calipers. Tumour volume was calculated according to the formula $V=ab^2\pi/6$, where a is the

longest diameter of the tumour and b is the longest diameter perpendicular to diameter a. From these volumes, tumour growth curves were constructed. Mice euthanized when the tumour diameter was between1.5cm.

3.4.3 *In vivo* Treg cell depletion

The anti-CD25 mAb (clone PC-61) was used to deplete and deactivate Treg in mice. Mice were treated with anti-CD25 mAb by i.p. injection in 0.25ml endonucleas-free PBS 1 day prior to performing experiments and every 4 days post tumour induction. Rat IgG1 (HRPN, anti-peroxidase horseradish) was used as a control (Bio-Express, NH). In previous studies the minimum dose required for each mAb was $1mgkg^{-1}$ delivered intraperitonealy (i.p.) to achieve >95% reduction in systemic Treg cell numbers without non-specific T-cell activation [20]. Validation that the control Ab had no effect on Treg cell numbers was achieved using FACS.

3.4.4 Flow cytometry analysis

FACS analysis was performed using anti-mouse PE-foxp3, FITC-CD4 and APC-CD25 and relevant isotype controls as per manufacturer (eBiosciences, Insight Biotechologies, UK). Single-cell suspensions from the spleens and tumours of individual mice were prepared to obtain a final concentration of 5×10^5 cells per well in blocking buffer (1× PBS/1% BSA/0.05% sodium azide/1% rat, hamster, and mouse serum). 50µl of each mAb dye and 5µl of the amine-reactive viability dye ViViD (Invitrogen) to determine dead cells was added to the blocking buffer. This was then incubated in the dark at 4°C for 30 min. Cells were washed twice with blocking buffer and finally re-suspended in 200µl 1% paraformaldehyde. To perform flow cytometric analyses and measure relative fluorescence intensities a FACS-Aria cytometer and BD Diva software (BD Biosciences) were used. For each mouse, 20,000–200,000 events were recorded. The percentage of cells labelled with each mAb was calculated in comparison with cells stained with isotype control Ab. Background staining was controlled by labelled isotype controls (eBiosciences) and fluorescence-minus-one. The results represent the percentage of positively stained cells in the total cell population exceeding the background staining signal. For determination of intracellular foxp3 production, cells were then fixed and saponin permeabilised (Perm/fix solution, eBiosciences) and incubated with mAb or isotypic

control mAb. After 30 min cells were twice washed in permeabilisation buffer (eBiosciences) and then analysed by flow cytometry as described above.

3.4.5 *In vivo* Electrogenetherapy

When tumours reached approximately 100mm^3 in volume (5-7mm major diameter), mice were randomly divided into experimental groups and subjected to specific experimental protocols. The procedure was carried out under general anaesthesia, by intraperitoneal (i.p.) administration of 200µg xylazine and 2mg ketamine. The skin overlying the flank tumour was shaved. Fifty µg of plasmid DNA in 50µl sterile injectable PBS was injected into the tumour. Two-needle array electrodes (Cork Cancer Research Centre, Cork, Ireland) were inserted on either side of the marked DNA injection point 80 seconds after DNA delivery for electroporation. The distance between the electrodes was 4mm. In vivo electroporation parameters were: 1200V/cm 100µsec pulse length; 1 pulse and 120V/cm 20 msec; 8 pulses at 1 Hz were administered in sequence using the ePORE Gx (Cork Cancer Research Centre, Cork, Ireland) square wave generator. The high voltage pulse was used to induce electroporation in the cell membrane and the ensuing small voltage pulses were used to create an electrophoretic field to assist movement of the negative charged DNA plasmid across the cells.

3.4.6 Statistical Analysis

Experimental results were plotted and analysed for significance with Prism 4 software (GraphPad software Inc, CA, USA). $p< 0.05$ was considered significant.

3.5 Results

3.5.1 Suppression of primary tumour growth following administration of anti-CD25 mAb

Mice were injected intraperitonealy (i.p.) with anti-CD25 mAb (PC61) or rat IgG1 one day prior to subcutaneous injection of B16F10 cells and every 4 days post tumour inoculation (Figure 3.1A) [25]. Subsequent monitoring of growth demonstrated that tumours grew significantly more slowly ($p < 0.05$) in the presence of anti-CD25 mAb when compared to untreated mice that had received no antibody or isotype control (IgG) (Figure 3.1B). Irrelevant rat IgG mAb when administered at an equivalent dose and time had no significant effect on growth or survival when compared with untreated tumours. Survival was improved significantly ($p<0.02$) when compared to the untreated group and the irrelevant antibody treated group from day 19 to day 24 (Figure3.1C).

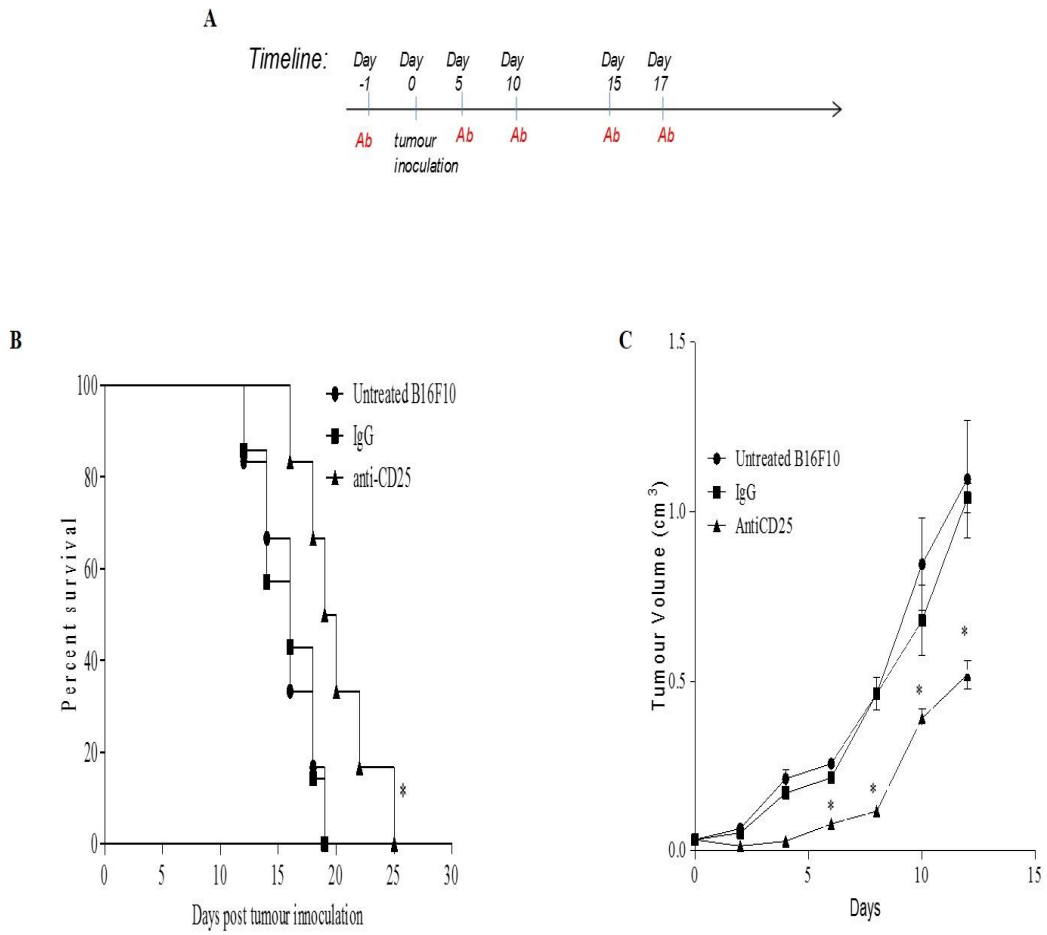

Figure 3.1: (**A**) Experimental protocol. C57/BJ6 mice (n=6) were treated i.e. with anti-CD25 mAb, irrelevant IgG mAb or untreated 1 day prior to tumour inoculation and 4 days post tumour inoculation, 5 x 10^5 B16F10 cells were inoculated subcutaneously to the mice and tumour volume was monitored. (**B**) Tumour growth curves. Tumour growth from anti-CD25 mAb, irrelevant IgG mAb and untreated is presented as tumour volume measurements from day 0 to day 12. Tumour volume was calculated using the formula $V=ab^2/6$. Data are presented as the means ± standard error of the mean. *At days 6, 8, 10 and 12, compared anti-CD25 mAb to untreated and IgG mAb, $p<0.05$. (**C**) Survival of tumour-bearing mice. Survival is presented using Kaplan-Meier survival curves. *Compared anti-CD25 mAb to untreated B16F10 and IgG mAb, $p<0.02$.

3.5.2 Immunogenetherapy Suppresses the Tumourigenicity of Murine Melanoma B16F10 Cells

B16F10 mouse melanoma is considered a poorly immunogenic tumour (ATCC). C57BL/6 mice were challenged with 5 x 10^5 B16F10 cells subcutaneously and subsequently electroporated with pGMCSF-B7.1, pMG or untreated when tumours reached approximately 100mm^3 in volume. Notably the immunogene group treated with pGMCSF-B7.1 showed significant reduction in tumour growth when compared to the untreated group (p<0.05), data presented (Figure 3.2A). This reduced tumour volume also correlated with significantly improved survival (p< 0.02), however the immunogenetherapy was not curative **(**Figure 3.2B).

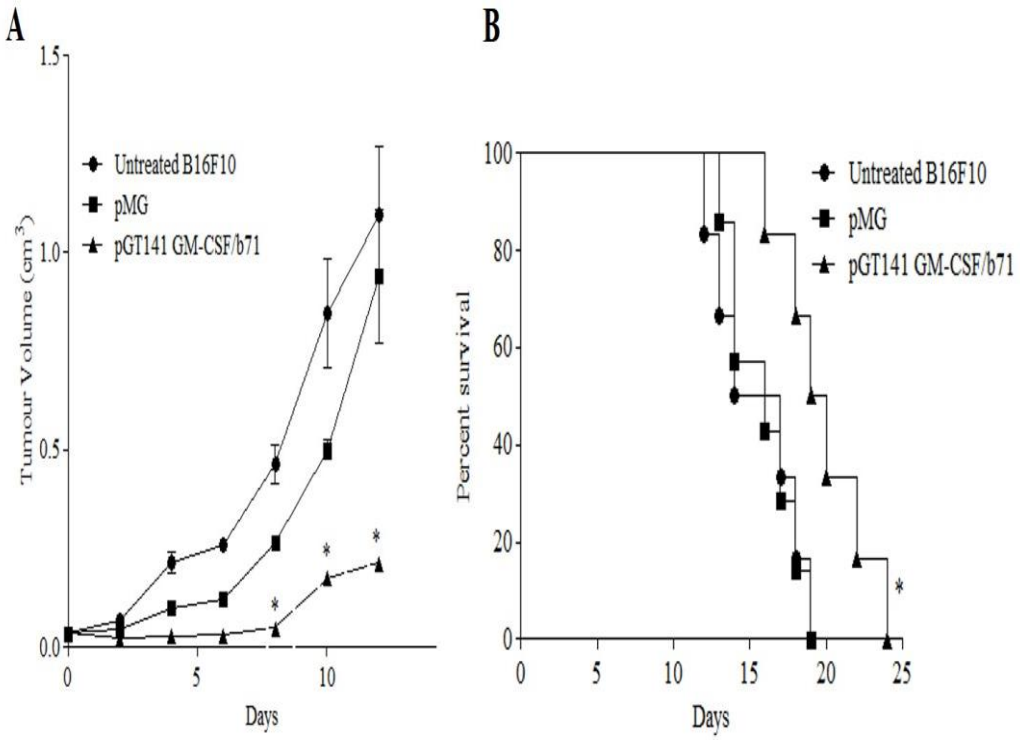

Figure 3.2: (**A**) Tumour growth of pGT141 GM-CSF-B7.1, pMG and untreated of pre-established B16F10 tumour is presented from day 0 to day 12. Data are presented as the means ± standard error of the mean. *At days 4, 6, 8, 10 and 12, compared pGMCSF-B7.1 to untreated B16F10 tumour, $p<0.05$. (**B**) Survival of immunogene treated tumour-bearing mice. Survival is presented using Kaplan-Meier survival curves. *Compared pGMCSF-B7.1 to untreated B16F10 tumour, $p<0.02$.

3.5.3 Combinational Therapy of anti-CD25 and Immunogene of B16F10 Primary Tumour

To determine how a combinational therapy approach might affect tumour growth we examined mice treated with a combination of immunogenetherapy and anti-CD25 mAb. Mice received either anti-CD25 or rat IgG1, B16F10 cells and were then electroporated with pGMCSF-B7.1, pMG or untreated when tumours reached approximately 100mm^3 in volume (Figure 3.3A). The pGMCSF-B7.1 and anti-CD25 mAb combination treatment regime significantly reduced the growth of the tumour when compared to the untreated B16F10 tumour (p<0.05). There was no difference in growth rate when pGMCSF-B7.1 and anti-CD25 combination group was compared to pGMCSF-B7.1 and IgG group and pMG and anti-CD25 group (Figure 3.3B). Importantly those mice receiving pGMCSF-B7.1 and anti-CD25 has significantly improved survival (p<0.05) compared to the untreated group (Figure 3.3C). Overall, the combinational therapy approach had similar primary tumour outcomes when compared to either modality alone. There was no improved in the combination over the single and modality treatments.

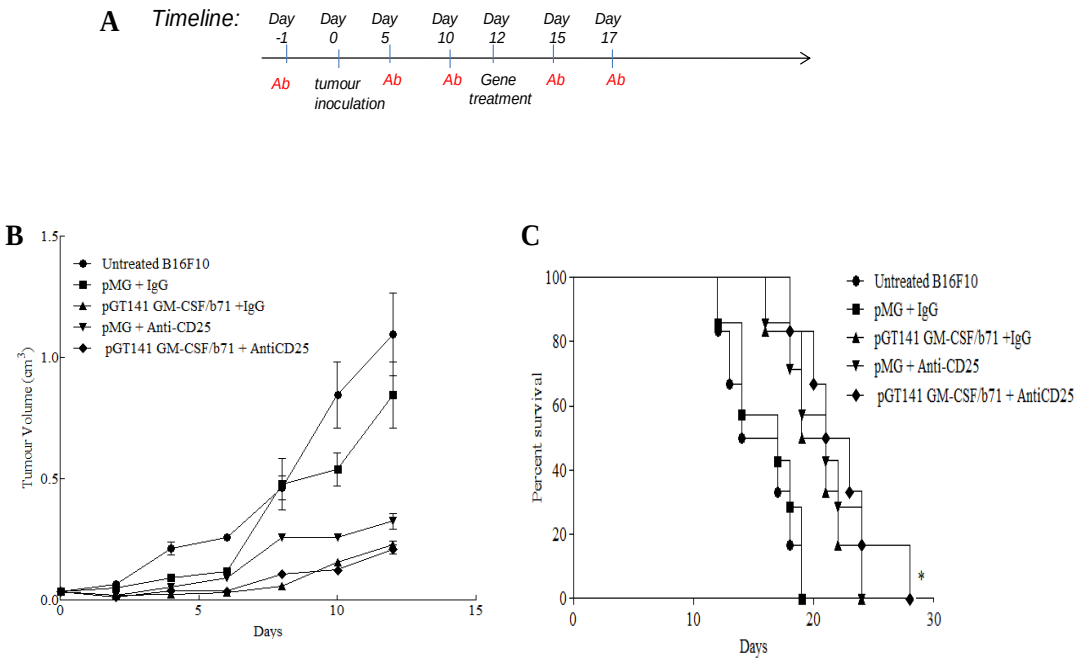

Figure 3.3: (**A**) Protocol of antibody administration, immunogenetherapy and tumour inoculation. C57/BJ6 mice (n=6) were treated using the antibody regime previously described and combined with immunogene when tumour reached approximately 100mm^3 in volume, 5 x 10^5 B16F10 cells were inoculated subcutaneously to the mice and tumour was monitored. (**B**) Tumour volume was measured and presented as means ± standard error of the mean. *Combinational treatment (pGMCSF-B7.1 and anti-CD25 mAb) compared to untreated B16F10, p<0.005. (**C**) Survival is presented using Kaplan-Meier survival curves. *Compared pGMCSF-B7.1/anti-CD25 mAb to untreated B16F10 tumour, p<0.02.

3.5.4 Suppression of Pulmonary Metastasis from B16F10 Tumour

We next sought to examine how effective the pGMCSF-B7.1 and anti-CD25 combination would be against metastatic disease. 24 days post primary tumour induction (Figure 3.4A), lungs were collected, weighed and photographed to determine the number of metastatic nodules on the lung surface (Figure 3.4B**)**. In untreated mice with a primary B16F10 tumour we observed approximately 265 metastatic nodules on the lung surface (Figure 3.4C). Mice in the single therapy groups (i.e. pGMCSF-B7.1 and IgG control or control pMG and anti-CD25) had significantly less metastatic nodules when compared to untreated or pMG and IgG combination ($p<0.05$). Notably only one mouse (out of 6) receiving the combination therapy (pGMCSF-B7.1 and anti-CD25) displayed any metastatic lung nodules and thus this group had significantly reduced ($p<0.05$) lung metastases when compared to single therapy or control groups.

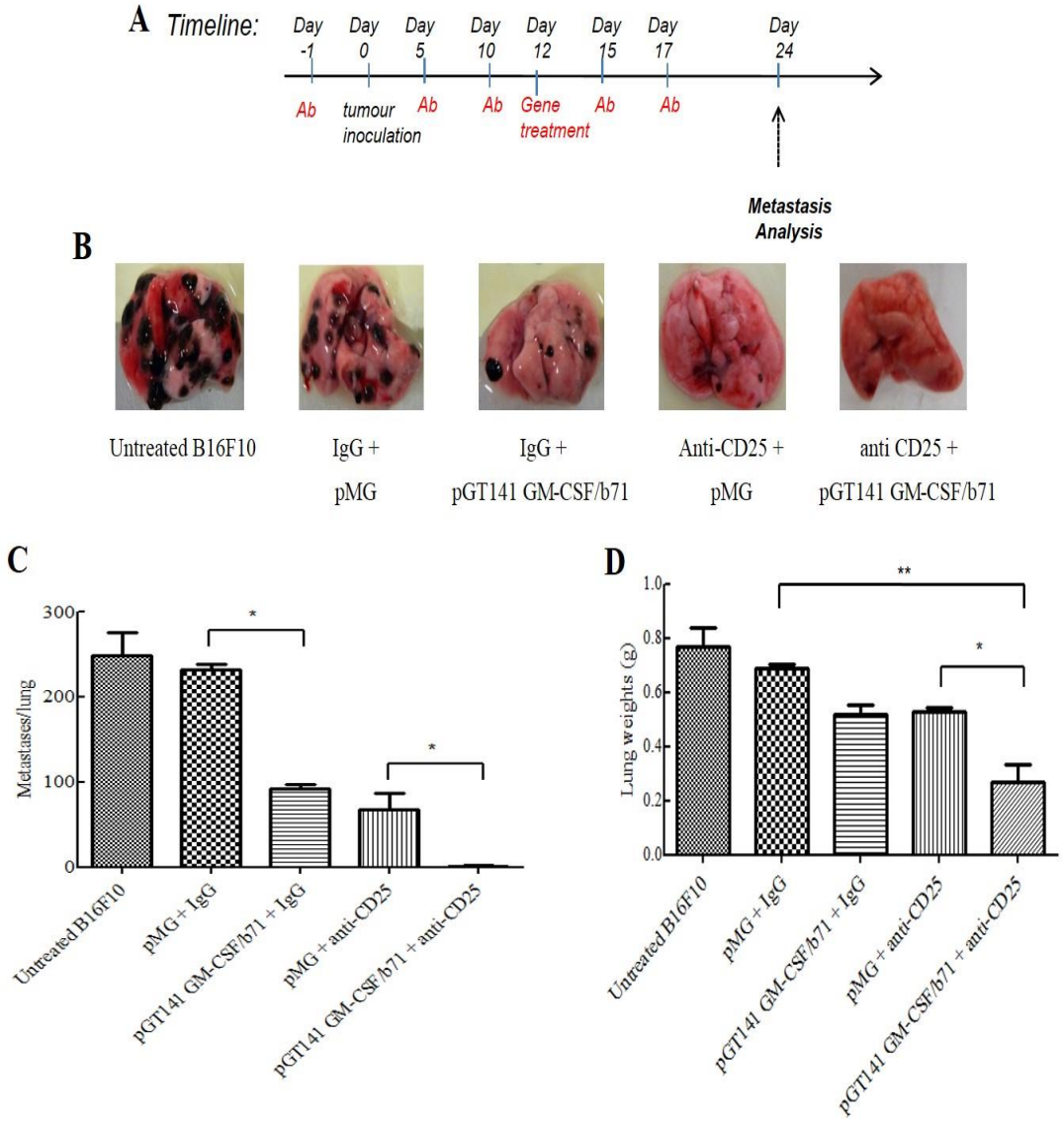

Figure 3.4*:* (**A**) Schematic representation of treatment schedule: C57/BJ6 mice (n=6) were treated i.e. with anti-CD25 mAb, irrelevant IgG mAb or untreated 1 day prior to tumour inoculation and 4 days post tumour inoculation, 5 x 10^5 B16F10 cells were inoculated subcutaneously to the mice and tumour volume was monitored. Tumours were treated with ± pGMCSF-B7.1 or pMG when the tumours reached approximately 100mm^3 in volume. (**B**) Macroscopic images of lungs (**C**) The number of surface metastases counted. The results represent those from 4 animals per group. (**D**) lung weight measurements are presented as means ± standard error of the mean. *pGMCSF-B7.1 and anti-CD25 mAb combinational treatment compared to pMG and anti-CD25, p<0.05. **pGMCSF-B7.1 and anti-CD25 mAb combinational treatment compared to pMG and IgG, p<0.05

3.5.5 T cell Changes During Treatment

In order to assess the effect the therapy had on T-cells systemically and locally in the B16F10 tumour model, we collected tumour and spleens from anti-CD25 mAb, pGMCSF-B7.1 and the combination of pGMCSF-B7.1 and anti-CD25 mAb treatment groups. As expected, the anti-CD25 mAb treatment group significantly depleted the $CD4^+CD25^+FoxP3^+$ cell population in both the tumour and spleen (Figure 3.5A-B). However there was no significant change in the $CD4^+CD25^+FoxP3^+$ tumour cell population in the local pGMCSF-B7.1 treatment (Figure 3.5C). The combination treatment with pGMCSF-B7.1 and anti-CD25 mAb reduced both the $CD4^+CD25^+FoxP3^+$ regulatory T cell and the $CD4^+CD25^+FoxP3^-$ T cell population (activated $CD4^+$ T cells) (Figure 3.5D).

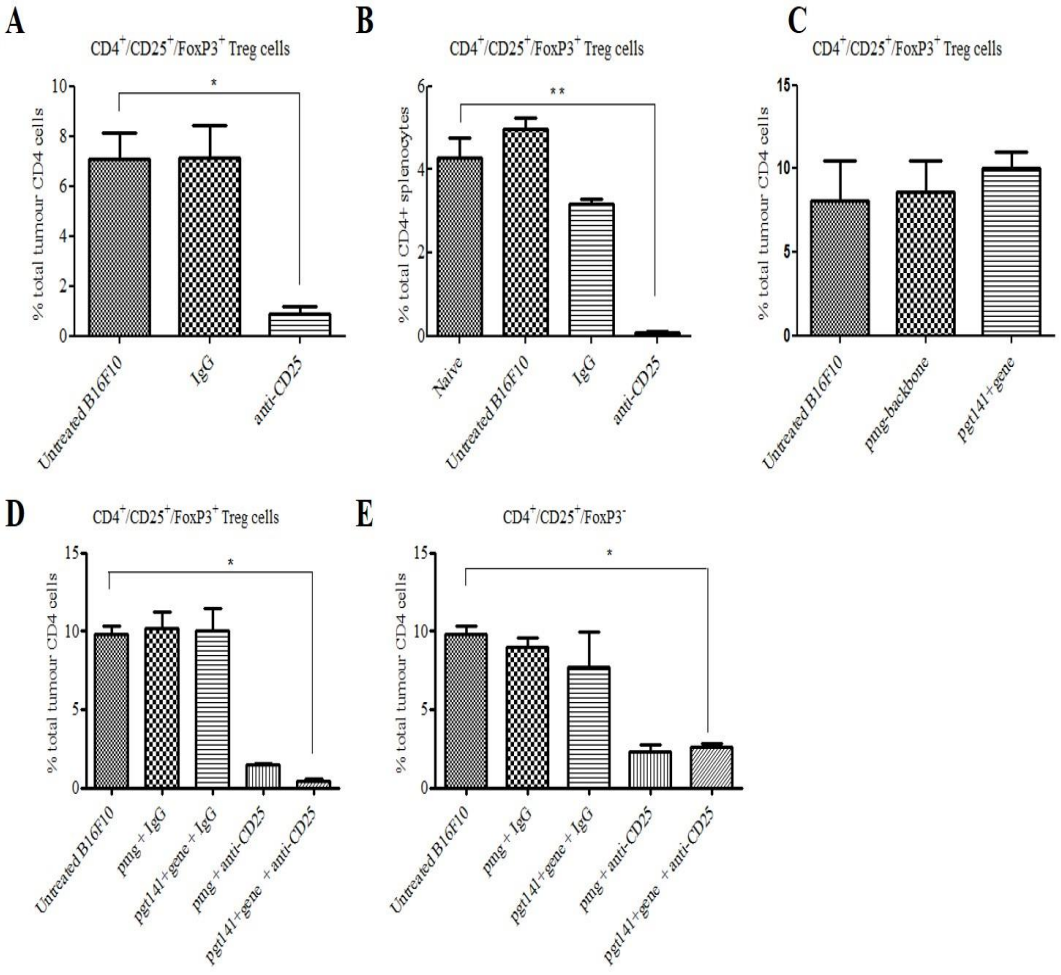

Figure 3.5: Anti-CD25 mAb i.p. administration inactivates Tregs locally and systemically at the tumour site and spleen. (**A**) The $CD4^+CD25^+FoxP3^+$ cell populations were significantly reduced. Compared anti-CD25 mAb to untreated B16F10 and IgG mAb in tumour $p<0.004$. (**B**) The $CD4^+CD25^+FoxP3^+$ cell populations were significantly reduced. Compared anti-CD25 mAb to untreated B16F10 and IgG mAb in the spleen $^{**}p<0.001$. (**C**) The immunotherapy with pGMCSF-B7.1 alone had no effect on the $CD4^+CD25^+FoxP3$ tumour cell populations. (**D**). Combinational treatment reduced the $CD4^+CD25^+FoxP3^+$ cell population, (**E**) Combinational treatment also reduced the $CD4^+CD25^+FoxP3^-$ cell population compared to the untreated tumour $^{*}p<0.05$. Data is presented as % respective cell population in the respective tissue ± standard error of the mean.

3.6 Discussion

Malignant melanoma is one of the most aggressive forms of cancers and despite the development of molecular targeted therapies and improvements in surgery and radiotherapy many patients succumb to recurrences and secondary's resulting in poor prognosis [9]. The absence of an effective immune response to metastatic melanoma is thought to contribute to this poor prognosis [11]. Several studies have described the presence and accumulation of Treg in tumours and their increase infiltrate to be associated to this overall poor prognosis. This accumulation increases with tumour volume and is coupled with the inhibition of innate immune rejection of the tumour and precludes the proliferation of effector cells [24-26]. Building on these observations we opted for a combinational regime of Treg depletion and an immunotherapy treatment.

We choose an in-house plasmid encoding the cytokine GMCSF and the co-stimulatory molecule human B7-1 which we previously demonstrated an effective recruitment of cytotoxic anti-tumour response and the permanent elimination of a poorly immunogenic fibrosarcoma tumour [20]. B7-1 is usually expressed on the membrane of antigen presenting cells (APC) whereas tumour cells usually lack its expression and without this costimulatory signal, T cells may become clonally anergic when the TCR signal is delivered. B7-1-transduced tumour cells are expected to present both the antigen (TCR receptor) and the costimulatory (CD28-mediated) signals to $CD8^+$ cytotoxic T lymphocytes (CTLs) simultaneously, leading to efficient activation of CTLs without requiring the assistance of $CD4^+$ helper T cells [24]. The potential of immune based cancer treatments has been limited by negative immune-regulatory mechanisms, including tumour-derived factors that support cellular immunity and also host factors that suppress/inhibit cellular immune responses. Immune-suppressive $CD4^+CD25^+FoxP3^+$ Treg accumulate in primary and metastatic cancers and can prevent treatment-induced immune responses therefore Treg depletion represents a promising therapeutic approach. Consistent with other studies we have shown that following anti-CD25 mAb administration, $CD4^+CD25^+FoxP3^+$ regulatory T cells were depleted significantly ($p<0.001$) with a minimal but significant improved survival probably due to the reduction in the metastatic load.

Previously we have demonstrated an improved local response in a range of tumour types treated by electrogenetherapy with a DNA plasmid encoding for the GM-CSF and the humanB7.1 (pGMCSF-B7.1) [20, 24]. In keeping with this trend, the administration of the pGMCSF-B7.1 was associated with extended survival in the B16F10 primary tumour without being curative. This observation could potentially be due to the level of Treg cells accumulating in the tumour subsequently blocking any additional immune recruitment, even with an optimal expression of GMCSF-B7.1. It is well documented that as a tumour grows the number of Treg cells also accumulates. Accumulations of Treg impinge on the effectiveness of any treatments and are associated with a negative clinical outcome. Thus, suppression of Treg so as to allow immune recruitment to the site of the tumour post pGMCSF-B7.1 expression would be a distinct advantage to any treatment approach.

We then decided to investigate the therapeutic potential of local pGMCSF-B7.1 combined with systemic anti-CD25 mAb against established subcutaneous tumour and lung metastasis derived from the weakly immunogenic B16F10 melanoma model. It was observed that there was a modest but significant improvement of primary tumour burden and survival observed for each of the individual treatments given alone or for the combinational treated groups. But the combinational therapy was not effective in halting the primary tumour. The aim of all anti-cancer therapies is long-lasting responses that also prevent metastases and secondary tumours. The combination regime had a remarkable therapeutic efficacy of pre-existing lung metastases when compared to the use of either treatment alone indicating the suppression of Treg cells with the anti-CD25 when combined with the immunotherapy pGMCSF-B7.1 allowed for an enhanced anti-tumour CD8 immune response with the decrease in the levels of metastatic nodules.

In conclusion, we have demonstrated that Treg depletion strategy combined with an immunotherapy was effective in our model. It was observed that the immunotherapy was only effective once the Treg cells were depleted. The combination of immumotherapy and Treg cell depletion resulted in an effective suppression of metastasis. We conclude the combined treatment was not effective in increasing the survival, but only effective in suppression of metastases. Although we were unable to stop the primary tumour this treatment could fit in as a debulking therapy prior to surgery and has the potential to treat the systemic and improving prognosis of metastatic melanoma patients.

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Chapter 4

Development and Characterisation of an Enhanced Non Viral Expression Vector (pEEV) for Electroporation Cancer Treatment

4.1 Summary

Despite advances in our understanding of the processes involved in the development and progression of cancer, treatment options for many patients are limited and prognosis still remains poor. Surgery followed by chemo- or radiation therapy has been the mainstay of treatment available for cancer patients. However, many cancers are resistant to these standard therapies. Non-viral plasmid DNA gene therapy represents a promising approach for the treatment of many diseases including cancer. Intracellular delivery of DNA can be achieved with the application of electroporation, facilitating the initial transport of exogenous DNA across the cell membrane into the cytoplasm. However, the variable rates of subsequent transport of DNA from the cytoplasm to the nucleus for mRNA expression has resulted in varying degrees of exogenous gene translation and proved a major limitation in comparison to viral approaches. To overcome these expression difficulties, we developed a proof of concept vector, the Enhanced Expression Vector (pEEV), incorporating elements from viral systems including nuclear localisation sequences and a viral replicase from the Semliki Forest Virus. The replicase allows for cytoplasmic mRNA expression and bypasses the need for nuclear localisation to generate high levels of gene expression. We have demonstrated that our 'Enhanced Expression Vector' – pEEV is capable of achieving high levels of expression in a variety of tissue types. The anti-tumour effects of pEEV were demonstrated by the delayed growth and increased survival of the non-therapeutic pEEV-treated CT26 tumour model. Using a novel endoscopic electroporation system, EndoVe, we demonstrate and compare for the first time both standard CMV promoter-driven plasmid and pEEV gene expression in intraluminal porcine tissues. The pEEV plasmid displayed reliable and superior expression capability and, due to its inherent induced oncolytic activity in transfected cells, may enhance the efficacy and safety of several cancer immune-gene therapy approaches.

4.2 Introduction

The field of cancer gene therapy is rapidly maturing and will no doubt be part of the future of cancer therapeutics. The critical questions are: Can any of these genes be used to induce cancer regression, increase sensitivity to conventional and biological anti-tumour treatments in cancer cells, or increase resistance of haematopoietic cells to cytotoxic agents?

The principal challenge for gene therapy lies in the development of a method of transfection that successfully delivers a therapeutic gene to selected cells where gene expression can be achieved. The ideal gene delivery vector should fulfill three criteria: 1) it should protect the therapeutic gene against degradation by nucleases in intercellular matrices; 2) it should safely deliver the therapeutic gene across the plasma membrane and into the nucleus of target cells; and 3) it should not be associated with any detrimental effects.

Clinical trials have thus far utilised two methods of gene delivery: viral and non-viral. Generally speaking, viral vectors offer an efficient means of gene transfection [168-171].

Despite the fact that viral vectors are able to mediate gene transfer with high efficiency and offer the possibility of long-term gene expression, they fail to fulfil the third criterion of the ideal gene therapy vector: safety [170, 172]. While retroviral vectors have the advantage of mediating stable gene transfer by integrating into the host genome and have a low potential for immunogenicity, this vector system has its limitations: 1) difficulties in producing high titres of retrovirus; 2) the fact that only actively dividing cells are capable of being infected; 3) the possibility of insertional mutagenesis [173-175].

Non-viral approaches with plasmid DNA offer potential advantages for clinical application particularly as they offer a reduced risk profile and a simplified preparation process. DNA plasmid vectors allow for the transfer of significantly larger genetic material than is possible using a viral system, are less expensive to manufacture, are considered safe, non-toxic and less immunogenic than viral vectors, and allow for repeat dosing if required [172, 176-178]. However, plasmid DNA vectors can often have lower expression profiles than viruses and therefore lack potency in human clinical trials [179]. Ultimately, for efficacy in gene therapy, the

plasmid must reliably express the gene of interest at adequate levels in the target cell [178, 179].

The technology of electroporation has been employed effectively in both preclinical and clinical settings for the delivery of plasmid DNA [126, 180, 181]. It has also been used to enable the uptake by passive diffusion of specific chemotherapeutic drugs, with very high reported anti-tumour efficacy and negligible side effects [181-184]. The process of electroporation involves the delivery of a microsecond pulse directly to the targeted tissue, which increases the local porosity of the tissue to macromolecules [39]. Electroporation has been established as a safe and effective method clinically with excellent responses observed in several cancer gene therapy studies. Therapies such as the electroporation delivery of plasmid DNA encoding for IL-12 and AMEP have advanced to clinical trials [185-188]. While electroporation facilitates the cytoplasmic absorption of plasmid DNA, it still lags behind viral methods for inducing a high degree of exogenous mRNA expression in the target cells. Therefore, it is important to establish and refine methods to improve electroporation based gene delivery for clinical use. Optimizing strategies include modulation of electric field strength and pulse duration to enhance plasmid delivery, alteration of the extracellular matrix with enzymatic and chemical methods and the introduction of reactive oxygen species (ROS) inhibitors to reduce plasmid DNA destruction by ROS generation post electroporation [189-193]. All have an impact on increased transfer and gene expression efficiency via electroporation.

Viruses ensure expression of their genome within an infected cell by - expressing a copy of their own replicase, which can transcribe copies of its viral genome within the cytoplasm. In general, the major concern for using virus as an expression vector is their infective nature, which involves biological risks [194]. However, the utilization of replication-deficient viral vectors such as Semliki Forest virus (SFV) avoids this problem while allowing rapid and high-level gene delivery [194]. The Semliki Forest virus is a positive-stranded RNA virus of the genus *Alphavirus* of the family *Togaviridae* [195, 196]. It is a relatively simple virus, encoding only nine functional proteins of unique sequence. The SFV replicon is a eukaryotic expression vector includes the cis-acting sequence for 5’ and 3’ ends of the virus genome and all the non-structural proteins (nsP), with the virus structural proteins replaced by the foreign gene [197, 198]. By utilising a DNA replicase copy from the Semliki Forest Virus and incorporating nuclear localisation sequences to

facilitate initial nuclear localization of the pEEV and replicase expression we have been able to overcome some of the drawbacks that have hindered the application of non-viral approaches to cancer gene therapy.

4.3 Study Aim

In this study we sought to deliver a non-viral vector system that has the potential for clinical application in electroporation based gene therapy. We report a proof of concept Enhanced Expression Vector (pEEV) which encompasses a viral replicase from the Semliki Forest Virus. We aimed to improve upon nuclear entry by the incorporation of a nuclear localization sequence. We demonstrated cytoplasmic expression of the vector. We also investigated the oncolytic capabilities of the pEEV. Furthermore we compared expression levels of the pEEV with standard available plasmids in murine and porcine animal models.

4.4 Materials and Methods

4.4.1 Plasmids

For plasmid transfection, a pCMV-lacz plasmid (Plasmid factory) was used encoding a β- galactosidase protein (LacZ) gene under the control of a cytomegalovirus (CMV) promoter (Figure 4.1D). The pEEV plasmid was created by incorporating a Semliki Forest virus DNA replicase sequence (kindly donated by Prof Greg Atkins, Virus Group, Department of Microbiology, School of Genetics and Microbiology, Trinity College Dublin). A nuclear localization sequence was also incorporated to allow for more efficient nuclear targeting of the pEEV (Figure 4.1B). The pMG plasmid was purchased from InvivoGen (Figure 4.1C). Plasmids were propagated in *E coli* strain Top10 and purified on endotoxin-free Qiagen-tip 500 columns (Qiagen).

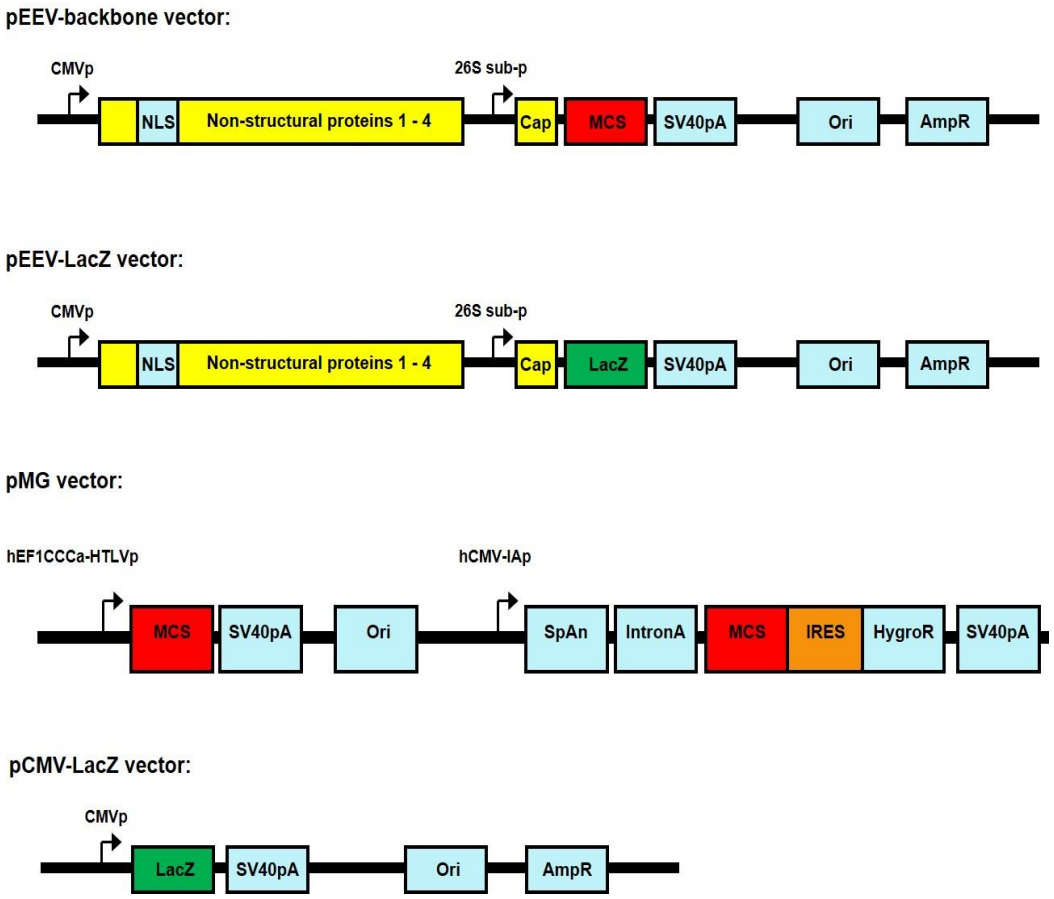

Figure 4.1 Schematic representation of the four circular plasmids used during this study **A.** pEEV-lacZ: The EEV plasmid was created by incorporating a Semliki Forest virus DNA replicase sequence (Non structural proteins 1-4), A nuclear localization sequence (NLS) was also incorporated to allow for more efficient nuclear targeting, a 26S subgenomic promoter (26S), the entire SFV capsid gene; which functions as a self-cleaving translation enhancer, capsid (CAP) gene, the LacZ gene which encodes for the β-galactosidase gene, SV40pA, an Ori colE1 (Ori) and an ampicillin resistance cassette (AmpR). **B.** pEEV: This is the backbone of the plasmid. The EEV plasmid was created by incorporating a Semliki Forest virus DNA replicase sequence (Non structural proteins 1-4), A nuclear localization sequence (NLS) was also incorporated to allow for more efficient nuclear targeting, a 26S subgenomic promoter (26S), the entire SFV capsid gene; which functions as a self-cleaving translation enhancer, capsid (CAP) gene, SV40pA, an Ori colE1 (Ori) and an ampicillin resistance cassette (AmpR). **C.** pMG: The pMG plasmid was purchased from InvivoGen. It incorporates the Human Elongation Factor-1α (EF-1α)/Human T-Cell Leukemia Virus Type 1 Long Terminal Repeat (HTLV) hybrid promoter (hEF1CCCa/HTLVp), a multicloning site (MCS), Simian virus 40 late polyadenylation signal (SV40pA). **D.** pCMV-lacZ: CMV promoter from pcDNA3, the LacZ gene which encodes for the β-galactosidase gene, SV40polyA, Ori and an AmpR.

4.4.2 Animal Studies

4.4.2.1 Ethics Statement

All animal husbandry and experimental procedures were approved by the University College Cork Animal Experimentation Ethics Committee and carried out under licenses issued by the Department of Health, Ireland as directed by the Cruelty to Animals Act Ireland and EU Statutory Instructions.

4.4.2.2 Mice

Female Balb/c, C57BL/6 and MF1-nu/nu mice (6-8 weeks) were obtained from Harlan Laboratories (Oxfordshire, England). All mice were kept in a conventional animal colony under standard conditions. Female mice in good condition, weighing 16-22g and of 6-8 weeks of age were used in the experiments.

4.4.2.3 Pigs

Female Pigs (Landrace) were obtained from Teagasc, Moorepark, Ireland and used at 25-30 Kg. Standard laboratory food and water were provided *ad libitum*. Pigs scheduled for surgery were fasted from the night before. Prior to initiation of general anaesthesia, pigs were sedated with 400mg of Ketamine and 80mg of Xylazine injected into the cervical musculature. Once the pig was sedated (approximately 5 minutes later), the skin overlying the flank and abdominal wall were shaved and thoroughly cleaned. A 22G cannula was inserted aseptically into a peripheral ear vein and flushed with normal saline to ensure correct positioning. 150mg/3ml of Amiodarone hydrochloride (a Class III anti-arrhythmic) was then administered intravenously (i.v.) as a cardioprotective agent. Anaesthesia was maintained using a 200mg Ketamine and 10mg Midazolam. This combination of sedation followed by maintenance anaesthesia resulted in approximately 40 minutes of deep general anaesthesia.

Prior to surgery, the level of anaesthesia was confirmed using the toe-pinch procedure. The porcine tissues accessed for study were the muscle, liver, spleen, ooesophagus and rectum.

4.4.3 Cell Tissue Culture

Tumour cell lines were obtained from the American Type Cell Collection (Manassas, VA). The murine colon adenocarcinoma, CT26 cell line was cultured with Dulbecco's modified Eagle's media (DMEM; Sigma), supplemented with 10% v/v foetal calf serum and 300µg/ml L-glutamine. The human adenocarcinoma, OE19 cell line was cultured in RPMI-1640 (Sigma) supplemented with 10% v/v foetal calf serum and 300µg/ml L-glutamine. Cells were maintained in logarithmic phase growth at 37°C in a humidified atmosphere supplemented with 5% CO_2.

4.4.4 Tumour Induction

For routine tumour induction, 1 x 10^6 CT26 or OE19 tumour cells, suspended in 200 µl of serum free DMEM were injected subcutaneously into the flank Balb/C (CT26) or MF1-nu/nu mice (OE19). Tumours were allowed to develop and size was monitored by measurements in two dimensions using Vernier callipers. Tumour volume was calculated according to the formula $V=ab^2\pi/6$, where a is the longest diameter of the tumour and b is the longest diameter perpendicular to diameter a. From these volumes, tumour growth curves were constructed. Mice were euthanized when tumour volume reached 1.7 cm^3 as approved by the University College Cork Animal Experimentation Ethics Committee.

4.4.5 DNA Transfection and *In Vivo* Electroporation

A range of tissues were transfected including a subcutaneous tumour (OE19) and healthy tissue (muscle, liver and spleen). Tissues were injected with 50 µg of the respective plasmid and surgically removed 48 hours following *in vivo* transfection by electroporation. The number of plasmid DNA and LacZ expression was then determined. Procedures were carried out under general anesthesia, using intraperitoneal (i.p.) .administration of 200 µg xylazine and 2 mg ketamine. The skin overlying the flank muscle and tumour was shaved and a midline laparotomy was used to expose the liver and spleen. Fifty µg of plasmid DNA in 50 µl sterile injectable phosphate buffer saline was injected into the tumour, muscle, liver and spleen. Parallel plate electrodes [130], with an adjustable separation setting, were inserted on either side of the marked DNA injection point and electroporation pulses

delivered within one minute of DNA injection. For this study plate electrodes were fixed at 1 cm apart.

Pigs scheduled for surgery were fasted from the night before. Prior to initiation of general anaesthesia, pigs were sedated with 400mg of Ketamine and 80mg of Xylazine injected into the cervical musculature. Once the pig was sedated (approximately 5 minutes later), the skin overlying the flank and abdominal wall were shaved and thoroughly cleaned. A 22G cannula was inserted aseptically into a peripheral ear vein and flushed with normal saline to ensure correct positioning. 150mg/3ml of Amiodarone hydrochloride (a Class III anti-arrhythmic) was then administered intravenously (i.v.) as a cardioprotective agent. Anaesthesia was maintained using a 200mg Ketamine and 10mg Midazolam. This combination of sedation followed by maintenance anaesthesia resulted in approximately 40 minutes of deep general anaesthesia. Prior to surgery, the level of anaesthesia was confirmed using the toe-pinch procedure. The porcine tissues accessed for study were the muscle, liver, spleen, ooesophagus and rectum.

Surgical access to muscle tissue was obtained by making a 6 cm linear incision over the appropriate flank using monopolar diathermy (Figure 4.2). Open surgical access to the liver and spleen was obtained through a midline laparotomy incision (Figure 4.3A and 4.3B).

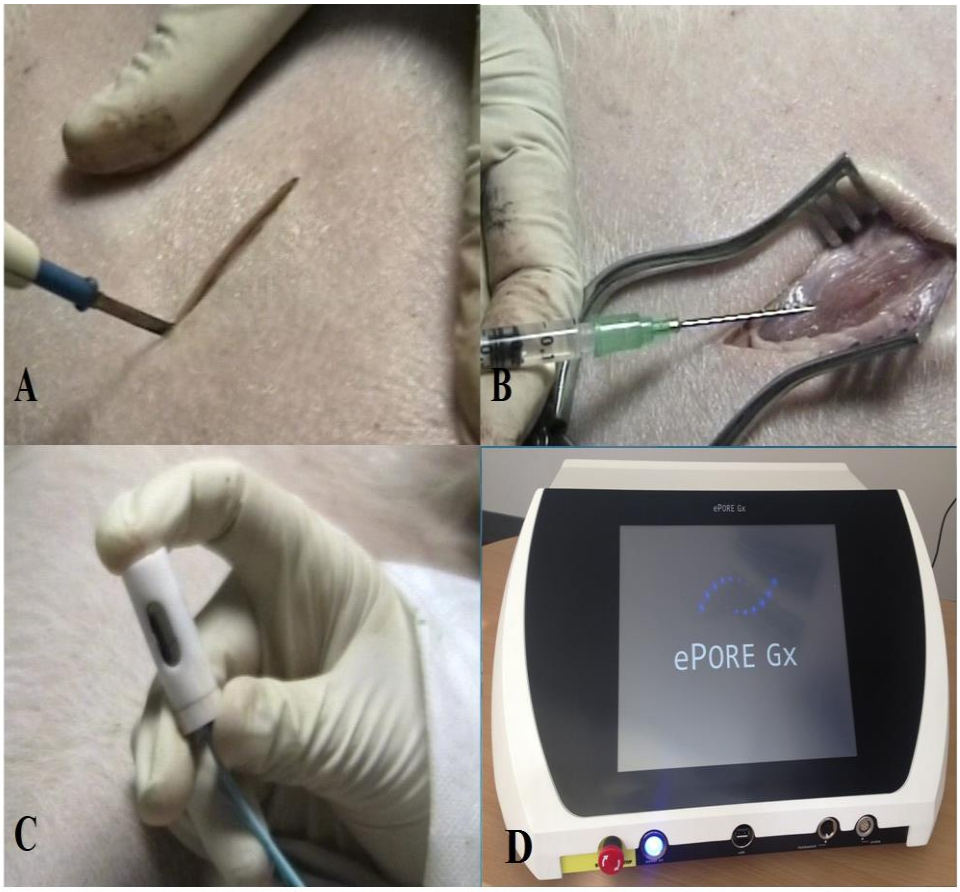

Figure 4.2 : (A) For surgical access to muscle, a 6cm linear incision was made using monopolar diathermy; (**B)** 50µg of plasmid DNA was injected intramuscularly (i.m.); (**C**) the EndoVe system of parallel plate electrodes was used to deliver the pulses; (**D**) Successful pulse generation was confirmed by the ePORE Gx (Cork Cancer Research Centre, Cork, Ireland).

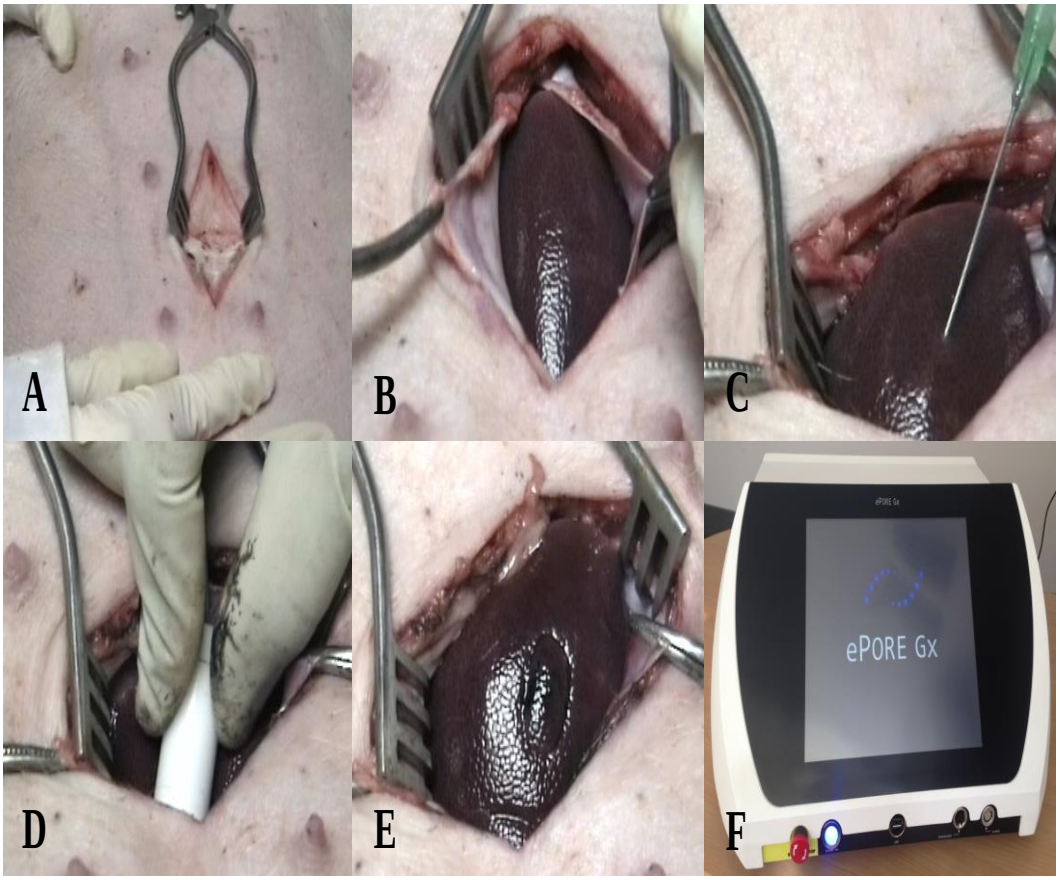

Figure 4.3: (A) For open surgical access to the liver and spleen, a midline incision was made using monopolar diathermy; (**B)** the liver (pictured) or spleen was exposed; (**C**) 50g of plasmid DNA was injected; (**D**) the EndoVe system of parallel plate electrodes were used to deliver the pulses; (**E**) successful vacuum suction of the tissue into the electroporation chamber was confirmed by the indentation left on the surface of the organ; (**F**) Successful pulse generation was confirmed by the ePORE Gx (Cork Cancer Research Centre, Cork, Ireland)

Fifty µg of plasmid DNA in 50µl sterile injectable phosphate buffered saline was injected directly into the target tissues (Figure 2.3C). Access to the gastrointestinal tissues - ooesophagus and rectum was achieved using the EndoVe device (Cork Cancer Research Centre, Cork, Ireland) (Figure 2.3D). Electric pulses were delivered using the EndoVe. *In-vivo* electroporation parameters were: A single 1200 V/cm 100 µsec pulses followed after one second by a train of 8, 120 V/cm 20 msec pulses at 1 Hz administered in sequence using the ePORE Gx (Cork Cancer Research Centre, Cork, Ireland) electroporation generator (Figure 2.3F).

4.4.6 Histologic Evaluation

Animals were sacrificed; tissues extracted and washed three times in PBS. β-galactosidase staining was performed on tissue using the β-galactosidase reporter gene staining kit (Sigma). Tissues were fixed in a 1X fixation solution for 30 min. After fixation, the tissues were rinsed three times in PBS and incubated for 24 h in X-gal staining solution. Tissues from each animal were processed separately. At the end of the incubation period, tissues were rinsed with PBS. Tissues were embedded in paraffin and serial sections were cut at 5 µm and mounted on slides. Heamatoxylin and Eosin staining was performed for both murine and porcine samples to assess for tissue damage.

4.4.7 RNA Isolation and Reverse Transcriptase Reaction

Frozen tissue samples preserved in RNAlater were homogenised in the presence of Trizol (Sigma) and total RNA was isolated according to manufacturer's instructions. Each sample was treated with DNase I (Ambion) to eliminate any possible DNA contamination and subsequently tested for presence of DNA using PCR amplification without the reverse transcription (RT) step. Total RNA concentration was determined from spectrophotometric optical density measurement (260 and 280 nm). For each sample tested, the ratio between the spectrophotometric readings at 260 nm and 280 nm (OD260/OD280) was used to provide an estimate of the purity of the nucleic acid, and the ratio in all samples ranged between 1.7 and 2.0. Reverse transcriptase reactions were carried out using the omniscript® reverse transcription kit (Qiagen) and Oligo$(dt)_{15}$ primer (Promega). Each reaction tube contained 1 µg of DNAse-treated total RNA in a volume of 20ul.

4.4.8 Genomic DNA Extraction

Genomic DNA was prepared according to manufacturer's protocol using the genElute™ mammalian genomic DNA miniprep kit (Sigma-Aldrich). Final DNA concentration was determined with the use of spectrophotometric measurement.

4.4.9 Real Time PCR (qPCR)

RT-PCR amplification was performed with the lightCycler® fastStart DNA master SYBR green I (Roche) at final concentrations of 3mM magnesium, 0.5 µM of each primer and 1 X LightCycler® FastStart DNA master SYBR green I. The final volume of each reaction was 20µl, containing 50 ng of RT product as template. The amplification programme started with 1 cycle at 95°C for 10 min, followed by 38 cycles at 95°C for 15 sec, 60°C for 20 sec and 72°C for 20 sec. The programme ended with 1 cycle at 72°C for 15 min. The following primers for lacZf and lacZr were designed to amplify 185-bp and their sequences are as follows lacZf 5'-GCG TGG ATG AAG ACC AGC-3', lacZr 5'-CGA AGC CGC CCT GTA AAC-3'. The housekeeping 18s rRNA was used as an internal control [199]. 18srRNAf 5'-TTG ACG GAA GGG CAC CAC CAG-3', 18srRNAr 5'-GCA CCA CCA CCC ACG GAA TCG-3'. For genomic PCR all samples were normalized to 100 ng/ µl of gDNA. 100 ng of DNA was used with similar PCR mixture contents and cycling parameters (40 cycles). LacZgf 5'-GAC GTC TCG TTG CTG CAT AA-3' LacZgr 5'-CAG CAG CAG ACC ATT TTC AA-3'.

4.4.10 Quantification of Apoptosis by TUNEL

To assess whether pEEV vector could also induce apoptotic death *in vivo*, pEEV, pEEV lacZ and pCMV lacZ was delivered into CT26 tumours via electroporation. Evaluation of the tumours for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL; Roche) staining (which marks apoptotic cells) was utilised. Balb/C mice bearing CT26 tumours were subcutaneously injected with 50 µg of plasmid DNA followed by electroporation. Mice were culled on 24, 48 and 72 hours post treatment and tumours were removed for detecting in situ apoptosis by TUNEL assay. The TUNEL-positive cells were stained brown. TUNEL staining of tissues was performed according to the manufacturer's protocol with minor modifications. In brief, processed tissues were

embedded in paraffin, cut into 5 µm thick sections, placed on superfrost® plus slides and adhered by heating at 37°C for 12 hours. Slides were deparaffinized and rehydrated by successive incubations in xylene, absolute ethanol, and 70% ethanol. Nuclei in tissue sections were stripped of protein by treating with proteinase k (20µg/ml) for 20 min followed by four successive washes with phosphate buffered saline (PBS). Slides were incubated with TUNEL reaction mixture at 37°C for 60 min in a humidified atmosphere followed by three successive washes with PBS. Sections were incubated with horse-radish peroxidise (Roche) for 30 min, rinsed in PBS and stained with DAB substrate kit (Dako) according to manufactures' recommendations. A positive control was established by incubating tissue section with DNase (1µg/ml) after deproteination and peroxidase inactivation. Intense staining was observed in cells from the DNase treated sections. A negative control was established by incubating tissue sample with DAB after deproteination and peroxidase inactivation. No staining was observed.

4.4.11 Bioluminescence Imaging

Bioluminescence imaging was performed as previously described (15), with an IVIS 100 charge-coupled device imaging system (Xenogen, Alameda, CA). Bioluminescent signals were quantified by the creation of regions of interest (ROIs). To standardise the data, light emission from the same surface area (ROI) was quantified for each transfected animal. In addition, background light emission, taken from ROIs created on untransfected control animals, was subtracted from transfected test animals. Imaging data were analysed and quantified with Living Image Software 2.50 (Xenogen) and expressed as photons/second/cm^2.

4.4.12 Cytoplasmic Expression Assay

Cytoplasmic expression was assessed for both plasmid systems using a nuclear free cytoplasmic prep. The standard commercial rabbit reticulocyte lysate cell-free system (Promega) was used to characterise translation products from mRNA encoding T7 RNA polymerase. This preparation consisted of a cytoplasmic free of nuclear material lysate and mRNA encoding T7 RNA polymerase. Each plasmid was incubated at 30°C for a period of 2 hours. Luciferase expression was detected using the IVIS 100 charge-coupled device imaging system (Xenogen, Alameda, CA).

pCMV was added as a control plasmid to demonstrate the efficiency of pEEV expression in cytoplasm lystae when there is a mRNA encoding T7 RNA polymerase present and the requirement of the T7 promoter.

4.4.13 Statistical analysis

Experimental results were plotted and analysed for significance with Prism 4 software (GraphPad software Inc, CA, USA). $P < 0.05$ was considered significant.

4.5 Results

4.5.1 Construction of pEEV

The pEEV construct consists of the CMV IE/T7 promoter and SV40 polyadenylation (pA) (Figure 4.4). It includes the entire SFV capsid gene, which functions as a self-cleaving translation enhancer, an Ori colE1 (Ori) and capsid (CAP) gene. It also includes the SFV 1-4 non-structural components and an ampicillin resistance cassette. To this an expanded multiple cloning site (MCS) was incorporated for the inclusion of foreign transcripts. For our study, lacZ transcript was incorporated. A nuclear localisation sequence (NLS): CACATAACGGGAGGGCCGGCGGTTACCAGGTCGACGGATATGACGGCAGG was inserted to allow for better transport in the nucleus.

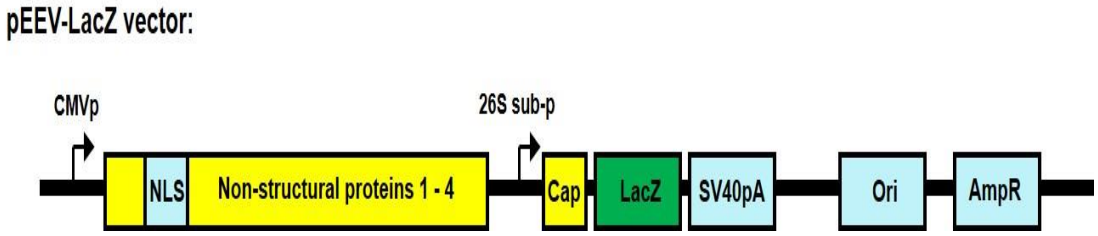

Figure 4.4: Schematic representation of the circular pEEV plasmid constructed for this study. The circular pEEV construct incorporates a CMV IE/T7 promoter, an SFV replicase (non-structural proteins 1-4), nuclear localisation sequence (NLS), the entire SFV capsid gene; which functions as a self-cleaving translation enhancer, an Ori colE1 (Ori), 26S subgenomic promoter, capsid (CAP) gene and an ampicillin resistance cassette (AmpR).

4.5.2 Comparison of pEEV-lacZ to Standard pCMV-lacZ

The pEEV plasmid (15.462Kb) is approximately three times larger, than the standard pCMV plasmid (6.233Kb), which could impact on transfection efficacy and expression. To establish this, we compared the transfection efficiency *in vivo* of pEEV with the pCMV plasmid. In order to determine efficiency, copy numbers of each plasmid expressing the lacZ transgene delivered by electroporation was measured. Both plasmids were detected in all tissues tested. In general, there were significantly more ($p<0.001$) copies of pCMV than pEEV (Figure 4.5), with the spleen and tumour having highest plasmid transfection.

To evaluate expression, qPCR was used to detect lacZ transgene expression as a reporter system (Figure 4.6**)**.

Transgene expression from the pEEV was significantly higher ($p<0.0001$), and on average 4 fold higher, than the respective pCMV plasmid. Taken together, these data indicate that high expression is achieved via an active vector, where, although present in fewer copies, pEEV allows an enhanced expression profile over the standard pCMV.

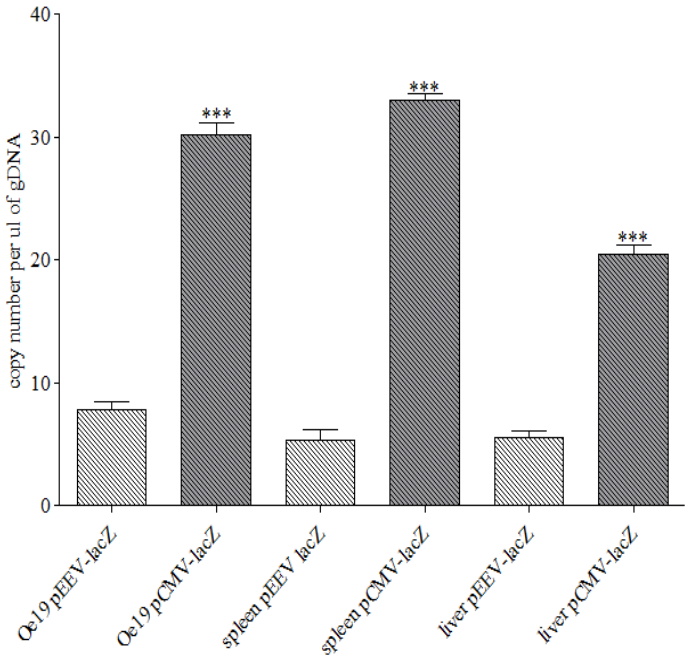

Figure 4.5: Bar graph showing absolute copy number of the lacZ transgene per ng of genomic DNA 2 days after electroporation obtained from MF1-nu/nu mouse tissue. All gDNA samples were normalised to 100ng of DNA prior to PCR. Each individual sample was analysed in triplicate for each qPCR. qPCR values are means ± standard error of the mean (SEM) of triplicate measurements. A comparison of pEEV (light grey bars) and pCMV (dark grey bars) samples were performed ***p<0.0001.

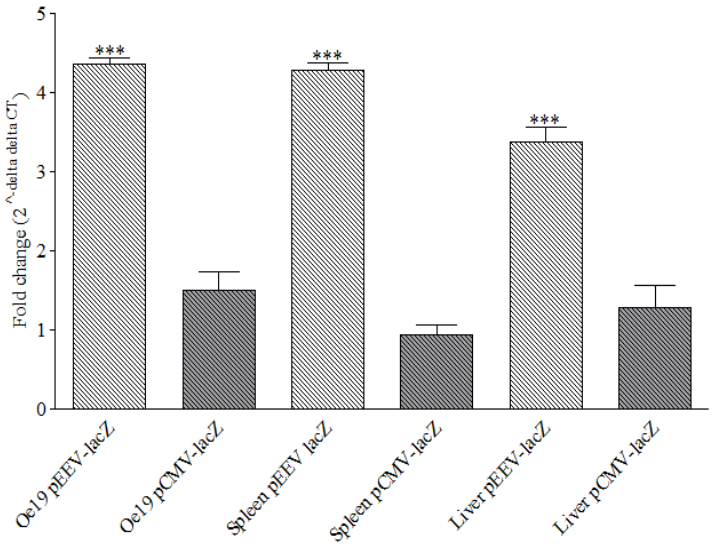

Figure 4.6: Bar graph presenting the relative expression of the lacZ mRNA 2 days after electroporation obtained from MF1-nu/nu mouse tissue. All qPCR data was normalised using 18S RNA as reference gene. Relative expression levels are plotted as means ± standard error of the mean (SEM) of triplicate measurements. pEEV (light grey bars) and pCMV (dark grey bars). pEEV lacZ expressed was significantly higher than pCMV lacZ ***p<0.0001.

4.5.3 Demonstration of Cytoplasmic Expression of Luciferase

The process for transcription of exogenous DNA material requires its translocation from the cytoplasm to the nucleus. The transcribed mRNA is then transported out of the nucleus for protein synthesis. Given that pEEV contains its own replicase, translation of the PEEV reported gene could occur within the cytoplasm. To assess the functionality of pEEV in a cell free assay, we used the functional activity of mRNA encoding T7 RNA polymerase, combined with the luciferase reporter gene. Luciferase expression from two plasmid constructs, the standard pCMV plasmid and pEEV were compared (Figure 4.7**)**. We could only detect pEEV driven luciferase expression during this assay. These data confirmed the ability of pEEV to self-express within the cell cytoplasm.

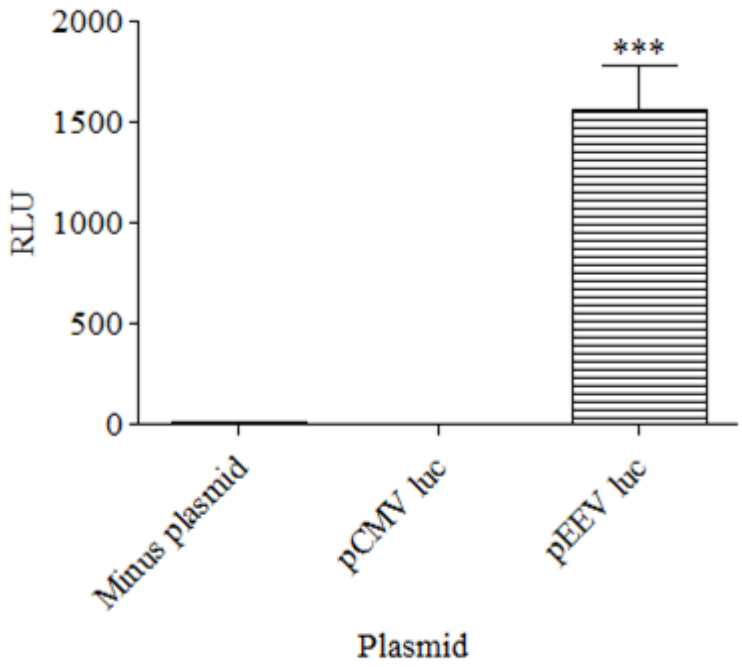

Figure 4.7: Luciferase reporter assay to verify activity of plasmid in a nuclear free preparation. Relative luciferase expression (RLU) was used as an indicator of expression and values are plotted as means ± standard error of the mean (SEM) of triplicate measurements. Only pEEV luciferase expression was detected.

4.5.4 Cytolytic Activity of pEEV

In our construction of pEEV, we utilized components of the SFV, we therefore hypothesized that the non-viral pEEV may delay tumour growth due to excessive gene expression inducing oncolytic activity, and leading to cell lysis of transfected cells. We transfected pEEV in an established tumour model to determine anti-tumour activity *in vivo*. In growing CT26 tumours pEEV lacZ, pEEV backbone, pCMV lacZ and pMG backbone were transfected by electroporation and growth and survival rates were examined and compared to its respective untreated CT26 tumour. The non-therapeutic bearing pEEV significantly ($p<0.05$) reduced tumour volume when compared to the untreated tumour (Figure 4.8).

Furthermore, we also observed improved survival of animal's transfected with non therapeutic pEEV. Interestingly, these collective data indicate that non-therapeutic pEEV can improve survival rates, which may be due to the oncolytic effect of pEEV lacZ (Figure 4.9).

To further demonstrate the oncolytic effect of non viral pEEV we transfected muscle of MF1-nu/nu mice and C57BL/6 with pCMV luciferase and pEEV luciferase (Figure 4.10).

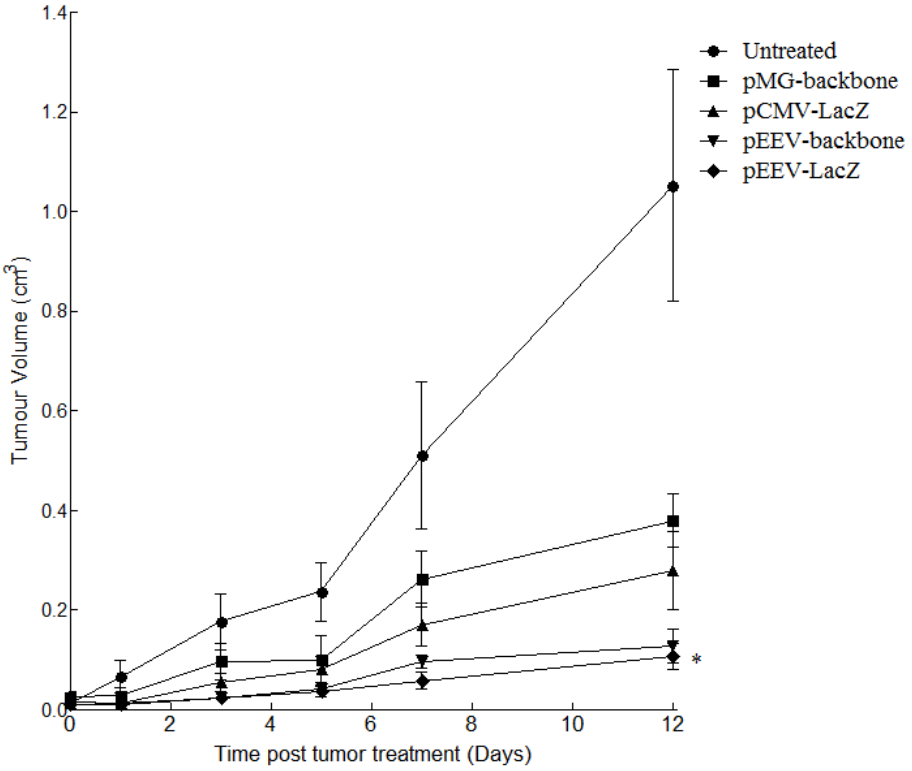

Figure 4.8: Tumour growth curve of CT26 tumours treated with pCMV backbone, pCMV lacZ, pEEV backbone, pEEV lacZ and an untreated tumours growing on Balb/c mice (n = 6 per group) is presented as tumour diameter measured. Tumour volume was calculated as previously described. Data presented are means ± SEM of 6 individual tumours. *Compared to the untreated group and pEEV lacZ, *p<0.05.

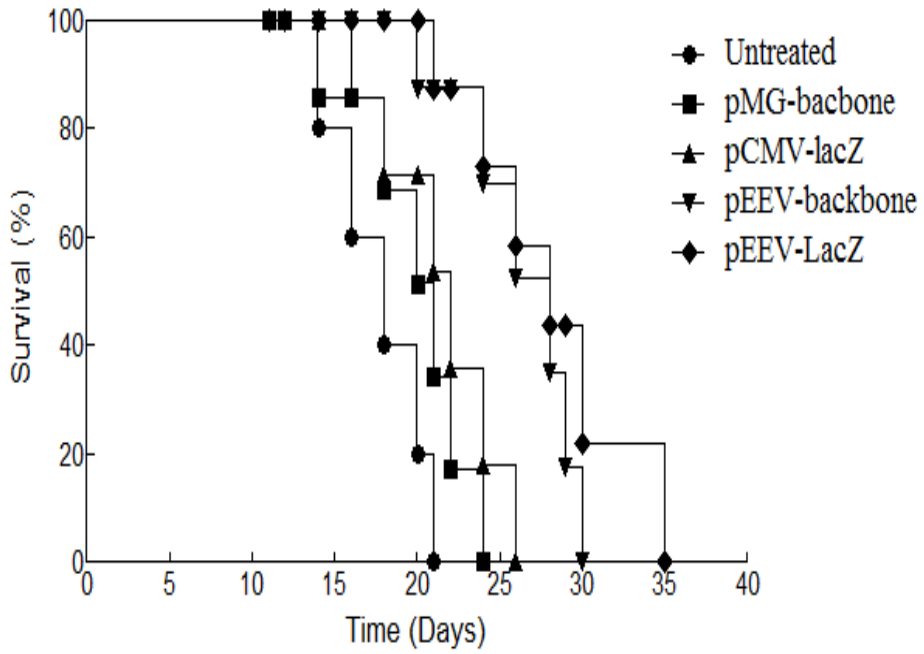

Figure 4.9: The respective survival curve of the CT26 treated tumours growing on Balb/c mice (n = 6 per group). The pEEV lacZ treated mice survived longer than any other group $p<0.05$ versus the untreated tumour. The pEEV lacZ group survived 4 days longer than the pEEV backbone.

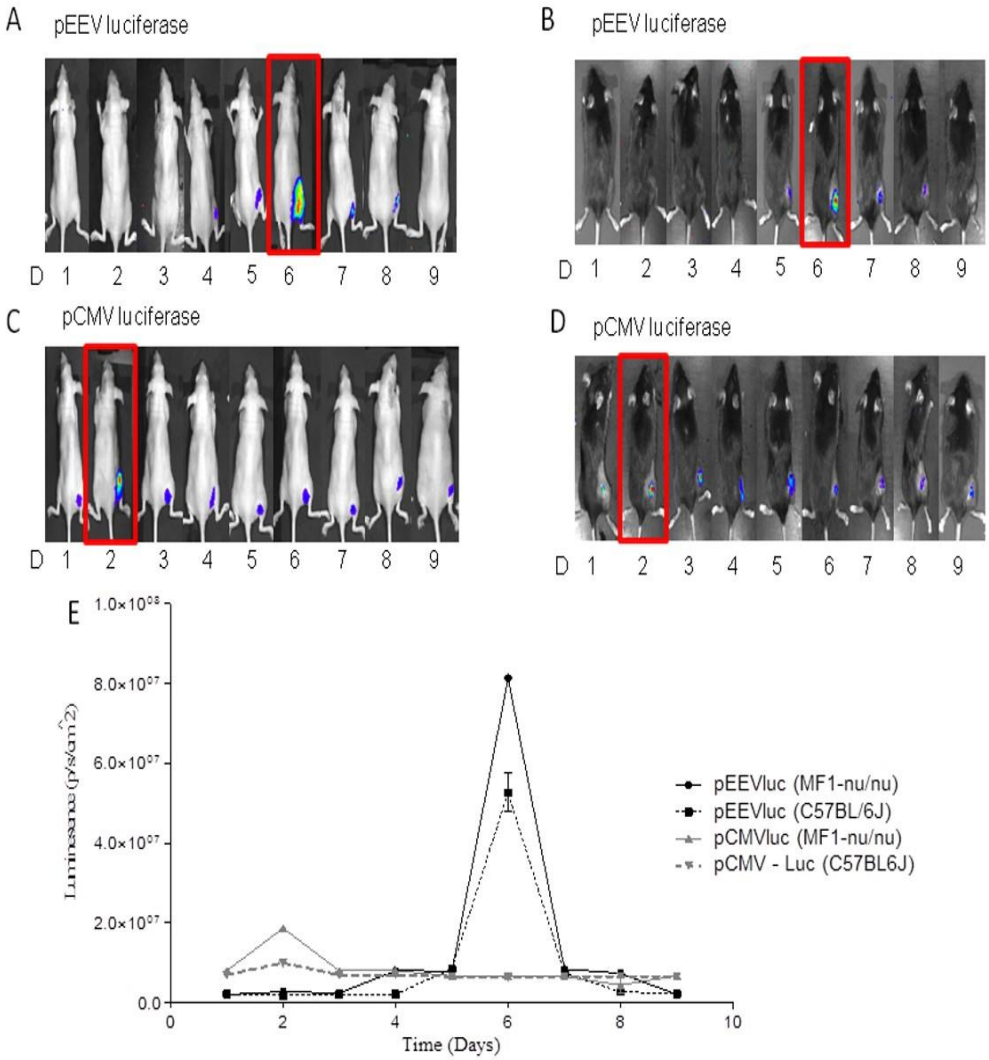

Figure 4.10: *In vivo* intramuscular (i.m.) plasmid delivery. *In vivo* muscle transfection by electroporation was demonstrated by luciferase activity, analyzed daly postintramuscular (i.m.) pCMVluc and pEEVluc, plasmid transfection via electroporation and subsequent gene expression was assessed using whole body imaging of luciferase expression. pCMV expression peaked day 2 in both C57BL/6J(C) and MF1-nu/nu mice (D) and pEEV peaked day 7 in both MF1-nu/nu (A) and C57BL/6J mice (B). No detection of pEEVluc was detected by day 9. (D) Bioluminesence data plotted over time for pEEVluc treated MF1-nu/nu mice (●) pEEVluc C57BL/6J mice (■) pCMVluc treated MF1-nu/nu mice (▲) and pCMVluc C57BL/6J mice treated mice (▼). Data is plotted as means ± standard error of the mean (SEM) of triplicate measurements Bioluminescent imaging revealed peak luciferase expression for pCMV at day 2 and day 6 for pEEV. pEEV expression was no longer detected by day 9, whereas, pCMV expression continued to be detected at low levels up until day 100. This quenching in luciferase expression of pEEV, suggests cell lysis due to the oncolytic effect of pEEV lacZ

4.5.5 Quantification of Apoptosis

To assess the ability of pEEV vector in induce apoptosis, we analysed in vivo electroporated CT26 tumours for TUNEL staining. TUNEL staining shown in (Figure 4.11) shows pEEV lacZ electroporation tumours were abundant in apoptotic nuclei, with double-strand DNA breaks, which are hallmarks of apoptosis. Notably, pCMV-treated tumours did not have any TUNEL staining (i.e. apoptosis). These data further confirm the oncolytic effect of pEEV.

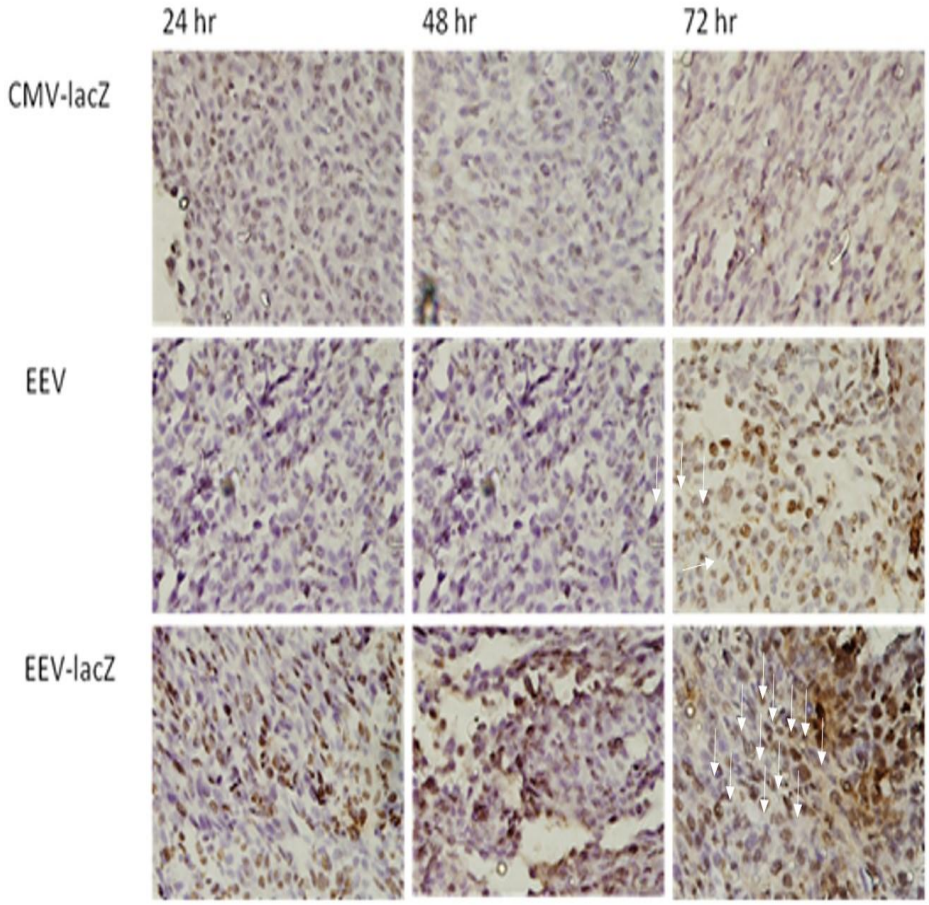

Figure 4.11: Apoptotic dead was evaluated using TUNEL staining on CT26 tumour model grown on Balb/c mice. Tumours were removed from mice at various time points following treatment; paraffin embedded and stained sections were examined microscopically. TUNEL stained for evidence of apoptotic cells indicate significant apoptosis in the tumour treated with pEEV lacZ and pEEV backbone but not from untreated groups.

4.5.6 β-galactosidase expression in a large animal model

Previous studies have indicated that DNA vaccines are often less effective in large animals when compared to responses induced in mice. Translation of plasmid expression into patients is essential for any therapeutic potential to be established. To determine if pEEV had this potential we tested the plasmid expression in a porcine model. To test transgene expression after electroporation, we examined LacZ transgene driven β-Galactosidase expression. Histological analyses of transfected tissues (Figure 4.12) demonstrated positive β-Galactosidase expression in all test tissues with all controls negative for β-Galactosidase expression. Importantly, the visual expression profile of pEEV lacZ was more abundant in comparison to the standard pCMV plasmid.

We again utilized the LacZ as a reporter transgene to compare the amount of gene expression induced from plasmids after electroporation, and to determine if the transgene expression was consistent with β-Galactosidase staining (Figure 4.13).

As observed in the mouse models, pEEV lacZ was expressed at a significantly higher level when compared to the standard pCMV lacZ. To investigate efficiency of transfection in our large animal model, we determined overall copy number (Figure 4.14).

Whilst we observed that overall copy number was significantly lower for pEEV when compared to pCMV (similar to murine studies), we again observed that pEEV gave a significantly stronger expression profile when compared to pCMV. Taken together, these results demonstrate that pEEV can be used to effectively express exogenous genes within multiple tissues in a large animal model.

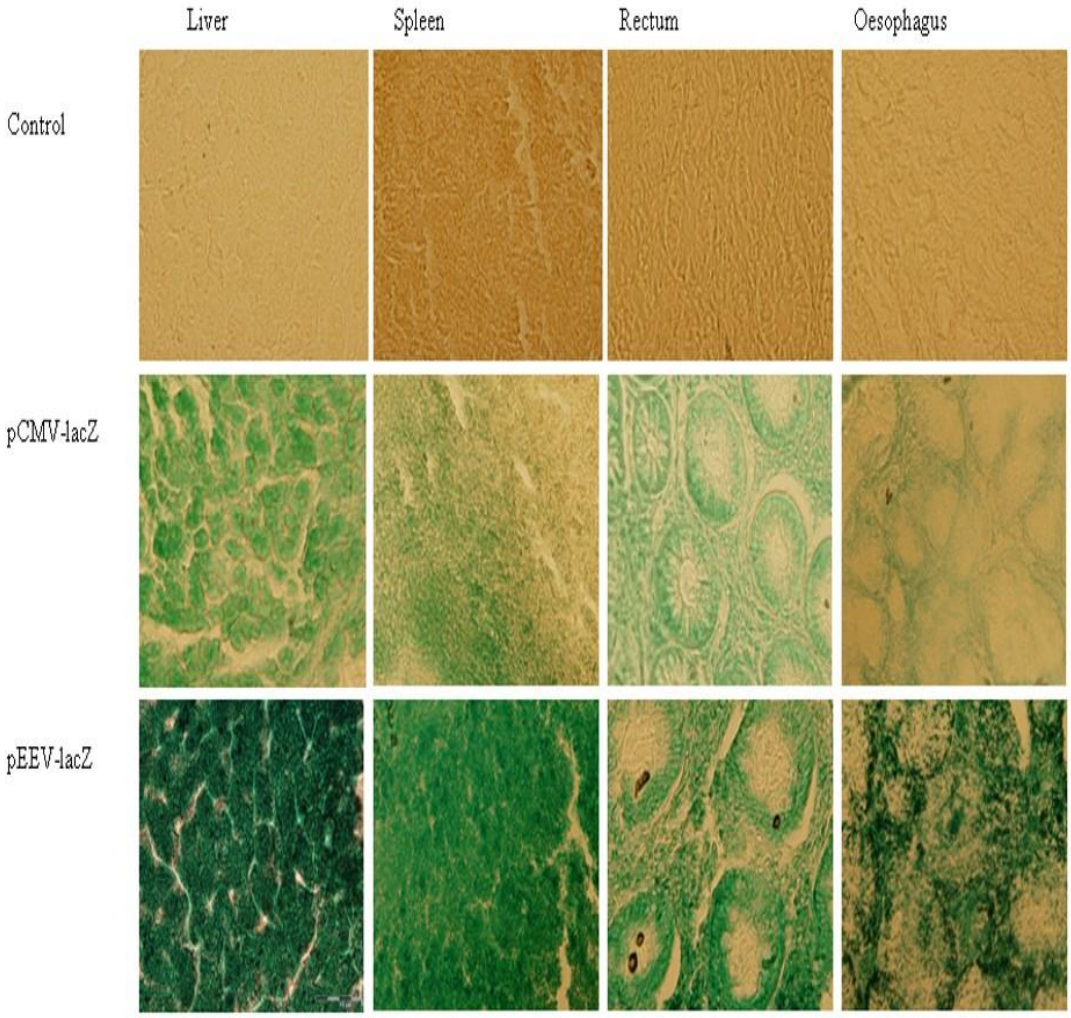

Figure 4.12: Representative image of β-galactosidase staining in porcine tissues. β-Galactosidase expression significantly increased in pEEV lacZ in all tissues been examined.

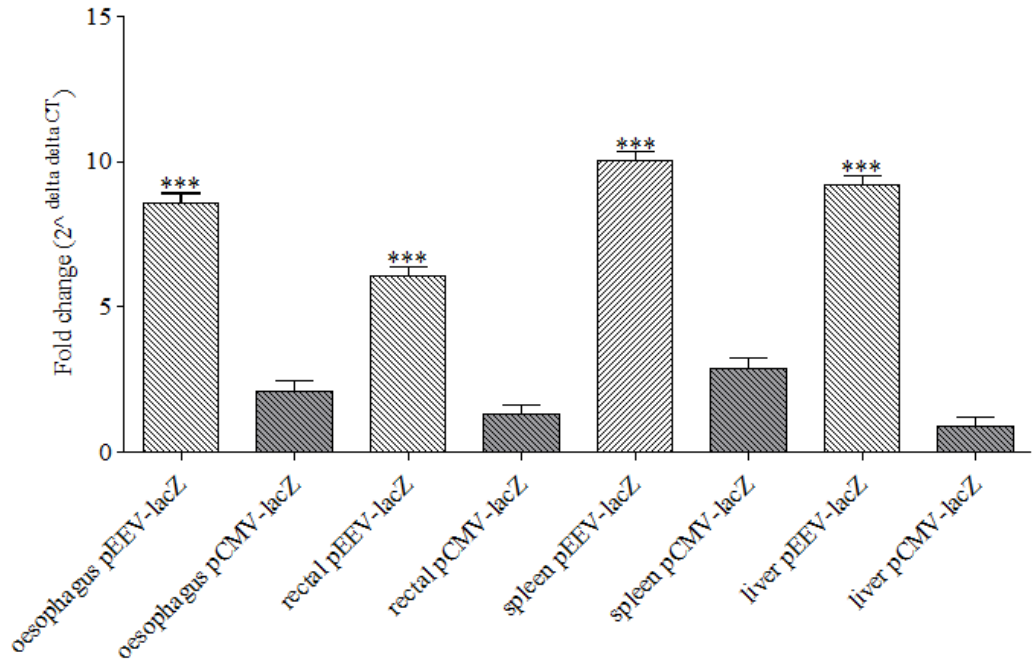

Figure 4.13: Bar graph presenting the relative expression of the lacZ mRNA 2 days after electroporation. All qPCR data was normalised using 18S RNA as reference gene. Relative expression levels are plotted as means ± standard error of the mean (SEM) of triplicate measurements. pEEV (light grey bars) and pCMV (dark grey bars). pEEV lacZ expressed was significantly higher than pCMV lacZ.

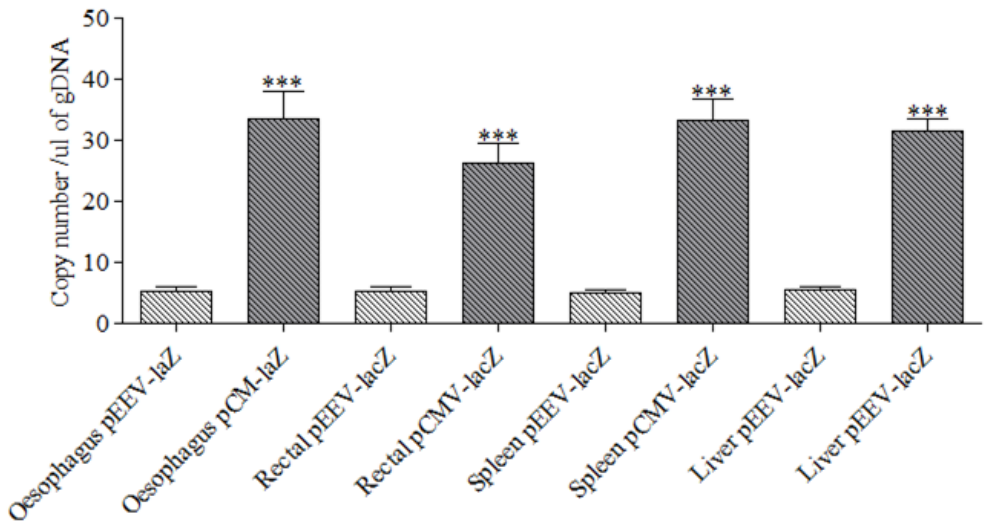

Figure 4.14: Bar graph showing absolute copy number of the lacZ transgene per ng of genomic DNAs 2 days after electroporation. All gDNA samples were normalised to 100ng of DNA prior to PCR. Each individual sample was analysed in triplicate for each qPCR. qPCR values are means ± standard error of the mean (SEM) of triplicate measurements. A comparison of pEEV (light grey bars) and pCMV (dark grey bars) samples were performed.

4.6 Discussion

The current study provides evidence for significant enhancements in the potential for non-viral DNA vector systems. Clinically non-viral-mediated transgene expression has received increased attention because it is a relatively safe, simple, and inexpensive compared to administration of viral vectors, which are associated with the induction of neutralizing antibodies and cytotoxicity [177, 194]. Electroporation is considered as a very useful tool for gene therapies and has received a lot of attention to development and improve in electrogene transfer protocols. Studies have included modulating the electric pulse, electrodes, extracellular matrix which has an effect on transfection efficiency and expression of the delivered gene [140, 189, 191, 192, 200]. Others have modulated the size of the plasmid being delivered and used plasmid types such as mini-circle-plasmid [201]. We have incorporated elements of a virus and nuclear localization sequence to allow for an enhanced expression of the gene of interest.

We have shown that a DNA based enhanced expression vector (pEEV), has improved expression capabilities across a range of tissue histologies over standard non- viral DNA vectors. pEEV was developed utilizing the Semiliki Forest Virus replicase, which allows for high levels of cytoplasmic expression and thereby overcoming the requirement and rate limiting step for all plasmid copies to enter the nucleus in order for mRNA transcription to occur. In the present study we evaluated our pEEV construct and demonstrated its superior efficacy using reporter gene analysis while comparing findings to a standard plasmid. pEEV delivered via electroporation, led to rapid mRNA expression in cancer cells. This approach was also found to have several advantages specifically for cancer therapy including; greatly improved and consistent gene expression, eventual direct oncolytic effect (cellular exhaustion) from continued mRNA production, which obviates the risk of long term exogenous gene expression.

The enhanced expression vector construct contains a DNA sequence 15Kb is size. There was initial concern that the large size of pEEV would impact negatively on its transfection efficacy after electroporation. Our data indicates that transfection levels for pEEV i.e. copies of plasmid DNA within the cell post electroporation, were indeed lower in comparison to the standard pCMV plasmid which was only 6.2kB in size. However, previous studies have indicated that self-amplifying vectors

only need a single RNA copy to reach the cytoplasm, whereby they can generate high expression levels almost independent of initial transfection and transcription efficiency. Indeed, although we observed overall lower transfection rates of pEEV, when compared to the standard pCMV plasmid, this did not however result in a proportional decrease in mRNA expression levels within a nuclear free lysate. Significantly, mRNA expression levels were in fact 4-5 fold higher in pEEV transfected cells, when compared to the more efficiently transfected standard commercial pCMV.

Targeting tumours with oncolytic viruses has demonstrated a promising approach for cancer treatment. These are modified or naturally oncotropic viruses, which selectively replicate in malignant tumour cells and finally destroy them via oncolysis but this approach also encounteres similar disadvantages as other viral therapies. Alphaviruses, like the Semliki Forest virus (SFV) have also been successfully used as oncolytic agents in several preclinical models of cancer. They are enveloped viruses containing a single positive strand RNA genome which, after, can replicate in the cytoplasm. The SFV system is suicidal and are characterised by their high expression levels. This process induces a strong cytopathic effect that leads to cell death by apoptosis. Taking these characteristics into account we incorporated these elements from the Semliki Forest Virus to develop the pEEV. We demonstrated cytoplasmic expression of pEEV and wanted to further characterise if pEEV had oncolytic effects like its viral parent. The stable presence and expression of plasmid DNA vectors in muscle have been demonstrated in several studies. We observed that *in vivo* muscle expression peaked on day 6 for pEEV transfection with expression levels declining and undetectable by day 9. This cellular oncolysis feature of pEEV could benefit cancer gene therapy applications such as by acting as a safety feature for over or continuous expression. Anti-tumour effects of pEEV were demonstrated by the delay in growth and increased survival of a non-therapeutic pEEV treated CT26 tumour model. This oncolytic characteristic and the high expression level trait of pEEV highlight the potential of this vector as an anti-cancer vector.

The electroporation gene transfer protocol appears to be safe and non-toxic. All mice remained healthy throughout the course of the experiments and there were no treatment-related deaths. This suggests that electroporation of solid tumours is not traumatic and that the levels produced by the pEEV did not induce systemic toxicity.

Other studies have observed similar safe and non-toxic effects of electrogene transfer [163, 202]. The ultimate aim of any preclinical gene therapy study is to translate findings to the clinic. The safety of electroporation as part of treatment in human patients has been validated in several studies of electrochemotherapy and electrogene transfer [186, 203].

We wanted to assess the of expression levels of pEEV, however efficacy in mouse models does not necessarily translate into patients. While our pEEV preclinical studies in mice revealed very promising results, we proceeded to validate the approach in the larger pig *in vivo* model. Pigs are an excellent larger animal model to study human physiology/disease due to many similar attributes including anatomy, genetics and physiology. Indeed, pig models have been used to evaluate the safety and efficacy of gene therapy; however studies have yielded varying results with regards to detectable levels of gene expression. Employing the EndoVe, a novel endoscopic electroporation device, we were able to demonstrate the reliability of the pEEV to express the b-galactosidase reporter gene across a range of intraluminal porcine tissue types. The high levels of pEEV reporter gene expression in this pig study indicates the potential utility of pEEV in the clinic and as a therapeutic tool in gene therapy. The data also clearly reveals the lack of consistent expression for the pCMV vector when translated from murine to porcine tissue, in contrast consistent levels of reporter gene expression were detected across all tissue types for the pEEV plasmid.

A growing number of clinical studies have established that electroporation is safe and effective in delivering plasmid DNA and chemotherapeutic drugs efficiently [185, 186]. In this study we used a previously reported safe and nontoxic parameters of electroporation-mediated transfer of the DNA plasmid to deliver pEEV *in vivo*. We found that the combination of an already established electroporation methodology and pEEV allows for an extremely reliable means of gene delivery and expression.

Taken together our results clearly demonstrate the potential of pEEV as a safe DNA vector with superior expression capabilities over standard available vectors. This vector, in combination with electroporation, allows for a safe and minimally invasive method of delivery. This study suggests that the pEEV expressing a therapeutic gene may have potential for clinical development with high detectable levels of expression.

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Chapter 5

Non-Viral Immune Electro-Gene Therapy Induces Potent Anti-Tumour Responses and has a Curative Effect in Murine Colon Adenocarcinoma and Melanoma Cancer Models

5.1 Summary

Anti-tumour efficacy of electroporated pEEV plasmid, coding for granulocyte-macrophage colony-stimulating factor and the B7-1 co-stimulatory immune molecule (pEEVGMCSF-B7.1) in growing solid tumours was investigated and compared to a standard plasmid. Application of pEEVGMCSF-B7.1 led to complete tumour regression in 66 % of CT26 treated tumours and 100 % in the B16F10 treated tumours at day 150 post treatment. pEEVGMCSF-B7.1 treatment was found to significantly enhance levels of both innate and adaptive immune populations in tumour and systemic sites, which corresponded to significantly increased tissue levels of pro-inflammatory cytokines including IFN-γ and IL-12. In contrast pEEVGMCSF-B7.1 treatment significantly reduced T regulatory populations and also the anti-inflammatory cytokine IL-10. Upon further characterisation of functional immune responses we observed a significant increase in cytotoxic (CD107a+) and IFN-γ-producing NK cells and also significantly more IL-12 producing B cells. Importantly, splenocytes isolated from pEEVGMCSF-B7.1 treated 'cured' mice were tumour-specific and afforded significant protection in a tumour re-challenge model (Winn assay). Our data indicate that electro-immunogene therapy with the non-viral pEEVGMCSF-B7.1 is able to induce potent and durable anti-tumour immune responses that significantly reduce primary and also secondary tumour growth and thus represents a solid therapeutic platform for future clinical trials.

5.2 Introduction

The current standard of care for cancer utilises surgery, radiation and chemotherapy, to achieve local tumour control and reduce the risk of disease recurrence [1]. Immunotherapy is potentially a new therapeutic pillar which can complement the current standard of care and can reduce risk of disease recurrence [2-4].

Immunotherapy based therapies have the potential to activate a tumour antigen specific response which can help to eradicate the tumour and reduce the risk of disease recurrence [5-8]. Delivering immunotherapies clinically can be achieved through a number of approaches including the use of gene therapy, which has many applications and methodologies already developed for cancer treatment [9-12]. For gene therapy to be successful, safe and efficient gene delivery is critical [11]. In current cancer gene therapy studies, viral vectors are used in the majority of gene delivery approaches, as they have high efficiency transduction [13, 14]. However, there are a number of significant drawbacks which include; efficiency of production, host immunogenicity, integration and safety [14, 15]. An alternative option to viral vectors is plasmid DNA. Toxicity is generally very low, and large-scale production is relatively easy [16]. However, a major obstacle that has prevented the widespread application of plasmid DNA is its relative inefficiency in gene [16, 17]. Therefore, most applications for plasmid DNA have been limited to vaccine studies, with a few exceptions [17, 18]. Consequently, methods that can significantly increase plasmid DNA transduction efficiency will greatly extend the utility of this promising mode of gene transfer. The technique of electroporation is widely used *in vitro* to effectively introduce DNA and other molecules into eukaryotic cells and bacteria. Application of short electrical pulses to the target cells permeabilises the cell membrane, thereby facilitating DNA uptake [19]. A number of studies, preclinical and clinical have shown highly successful responses with electroporated plasmid DNA encoding immune genes and also chemotherapeutic drugs [20-24]. Recently, we have shown that that applying electroporation to a range of tissue types utilising a new electroporation system, EndoVe in a large pig model. This will significantly enhance the application of electro-gene therapy [25].

Several cytokines have demonstrated significant anti-tumour effects. Among these, Granulocyte-macrophage colony-stimulating factor (GMCSF) is one of the most potent, specific and long-lasting inducers of anti-tumour immunity. GMCSF can mediate its effect by stimulating the differentiation and activation of dendritic

cells (DCs) and macrophages, increasing antigen presentation capability [26]. For optimal antigen presentation, engagement of the T-cell receptor (TCR) with an antigen/major histocompatibility complex requires the costimulatory molecule such as B7-1 (CD80) and B7-2 (CD86) [26, 27]. Subsequently, DC and macrophages, process and present tumour antigens to T cells, both CD4+ and cytotoxic (CD8+) T cells, augmenting the anti-tumour response [27]. As such, GMCSF is particularly effective in generating systemic immunity against a number of poorly immunogenic tumours [26].

5.3 Study Aim

We recently characterised a non-viral vector therapy system; pEEV with a vastly superior expression capacity when compared to a standard control vector. We present here the use of pEEV as a gene therapy vector carrying murine GMCSF and human b7-1 genes (pEEVGMCSF-B7.1). We used electroporation as a means to facilitate the delivery of the EEV plasmid and assessed the efficacy and immune induction in primary and secondary responses to treatment in murine colon adenocarcinoma and melanoma cancer models.

5.4 Materials and Methods

5.4.1 Cell Tissue Culture

Tumour cell lines B16F10, CT26, LLC and 4T1 were obtained from the American Type Cell Collection (Manassas, VA). The murine colon adenocarcinoma, CT26 and lewis lung carcinoma, LLC cell line was cultured with Dulbecco's modified Eagle's media (Sigma) supplemented with 10% v/v foetal calf serum, 300µg/ml L-glutamine. The human adenocarcinoma, 4T1 cell line and the mouse melanoma cell line was cultured in RPMI-1640 (Sigma) supplemented with 10% v/v foetal calf serum and 300µg/ml L-glutamine. Cells were maintained in logarithmic phase growth at 37°C in a humidified atmosphere supplemented with 5% CO_2.

5.4.2 Ethics Statement

All murine husbandry and experimental procedures were approved by the University College Cork Animal Experimentation Ethics Committee and carried out under licenses issued by the Department of Health, Ireland as directed by the Cruelty to Animals Act Ireland and EU Statutory Instructions.

5.4.3 Animals and Tumour Induction

Female Balb/c, MF1-nu/nu and C57BL/6J (6-8 weeks) were obtained from Harlan Laboratories (Oxfordshire, England). All mice were kept in a conventional animal colony under standard conditions. Female mice in good condition, weighing 16-22g and of 6-8 weeks of age were used in the experiments. For routine tumour induction, 5 x 10^5 tumour cells suspended in 200µl of serum free DMEM were injected subcutaneously into the flank of the female C57BL/6J, Balb/C or MF1-nu/nu mice. Following tumour establishment, tumours were allowed to grow and develop and were monitored by alternate day measurements in two dimensions using Vernier callipers. Tumour volume was calculated according to the formula $V=ab^2\pi/6$, where a is the longest diameter of the tumour and b is the longest diameter perpendicular to diameter a. From these volumes, tumour growth curves were constructed. Mice euthanized when tumour diameter was between 1.7cm^3.

5.4.4 Plasmids

The pMG plasmid was purchased from InvivoGen. A version of this plasmid, designated pGT141, containing the murine GMCSF and human B7-1 genes transcriptionally controlled from the hEF1-HTLV and CMV promoters respectively was designed and cloning was performed on contract by Invivogen. The EEV plasmid was created by incorporating a Semliki Forest virus DNA replicase sequence (kindly donated by Prof Greg Atkins, Virus Group, Department of Microbiology, School of Genetics and Microbiology, Trinity College Dublin). A nuclear localization sequence was also incorporated to allow for nuclear targeting. The murine *GMCSF* and human *B7.1* genes was also incorporated into the pEEV. Plasmids were propagated in *E coli* strain Top10 and purified on endotoxin-free Qiagen-tip 500 columns (Qiagen) The four plasmids used in the course of this study are demonstrated in (Figure 5.1).

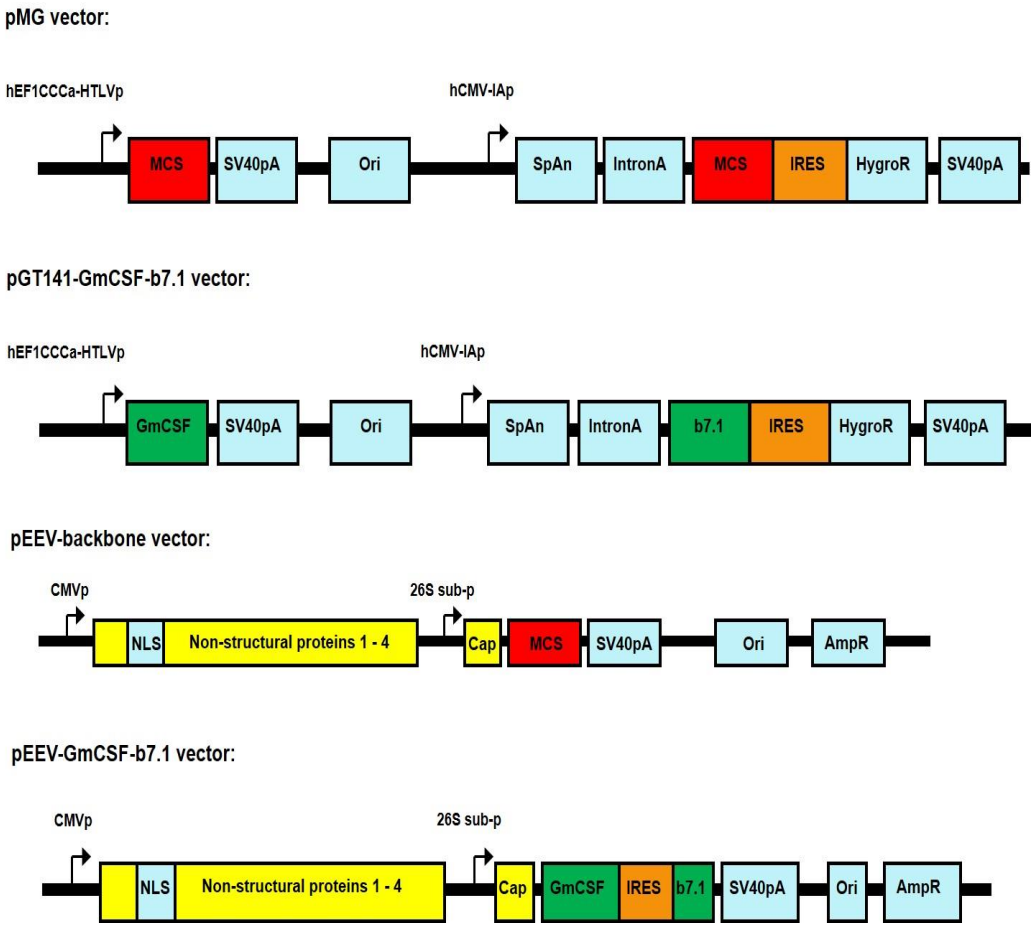

Figure 5.1: Schematic representation of the four circular plasmids used during this study **(A)** pEEV: This is the backbone of the plasmid. The EEV plasmid was created by incorporating a Semliki Forest virus DNA replicase sequence (Non-structural proteins 1-4), A nuclear localization sequence (NLS) was also incorporated to allow for more efficient nuclear targeting, a 26S subgenomic promoter (26S), the entire SFV capsid gene; which functions as a self-cleaving translation enhancer, capsid (CAP) gene, SV40pA, an Ori colE1 (Ori) and an ampicillin resistance cassette (AmpR). (**B)** pEEVGMCSF-B7.1: The EEV plasmid was created by incorporating a Semliki Forest virus DNA replicase sequence (Non-structural proteins 1-4), A nuclear localization sequence (NLS) was also incorporated to allow for more efficient nuclear targeting, a 26S subgenomic promoter (26S), the entire SFV capsid gene; which functions as a self-cleaving translation enhancer, capsid (CAP) gene, GMCSF-IRES-B7.1; coding for granulocyte-macrophage colony stimulating factor-internal ribosome entry site- B7.1 co-stimulatory immune molecule, SV40pA, an Ori colE1 (Ori) and an ampicillin resistance cassette (AmpR). (**C)** pMG: The pMG plasmid was purchased from InvivoGen. It incorporates the Human Elongation Factor-1α (EF-1α)/Human T-Cell Leukemia Virus Type 1 Long Terminal Repeat (HTLV) hybrid promoter (hEF1CCCa/HTLVp), a multicloning site (MCS), Simian virus 40 late polyadenylation signal (SV40pA). **(D)** pGT141GMCSF-B7.1: The pGT141GMCSF-B7.1 was purchased from InvivoGen. It incorporates the HEF1CCCa/HTLVp promoter, GMCSF, SV40, Ori, human CMV-IA promoter, SpAn, Intron A, B7.1, ECM IRES, hygromycin resistance cassette (HygroR). The GMCSF and B7.1 transcriptionally controlled from the HEF1CCCa/HTLVp and CMV-IA promoters respectively.

5.4.5 DNA Transfection and *In Vivo* Electroporation

Electroporation procedure was carried out under general anaesthesia, by intraperitoneal administration of 200µg xylazine and 2mg ketamine. Fifty µg of plasmid DNA in 50µl sterile injectable PBS was injected into the tumour followed by electroporation. *In vivo* electroporation parameters were: 1200 V/cm 100 µsec pulse length; 1 pulse and 120V/cm 20 msec; 8 pulses at 1 Hz were administered in sequence using the ePORE Gx (Cork Cancer Research Centre, Cork, Ireland) square wave generator. The high voltage pulse was used to induce electroporation in the cell membrane and the ensuing small voltage pulses were used to create an electrophoretic field to assist movement of the negative charged DNA plasmid across the cells.

5.4.6 Flow Cytometry Analysis

Single cell suspensions from spleens and tumours of individual mice were prepared. Cells were added at a concentration of 2 x 10^5 cells/well (96-well plate) in blocking buffer (1 x PBS/1% BSA/0.05% sodium azide/1% rat, hamster and mouse serum). To this; 50µL of each mAb dye mix was added plus 5µL of amine-reactive viability UV dye (Invitrogen) to determine dead cells, with incubation in the dark at 4^{o}C for 30 min [28]. The mAb used for flow cytometry are listed in Table 5.1.

Target Molecule	Clone	Isotype	Conjugate	Source
CD19	1D3	IgG2A	Alexa Fluor 700	eBiosciences
CD49b	DX5	IgM	FITC	eBiosciences
CD3	500A2	IgG	Alexa Fluor 700	eBiosciences
CD11c	N418	IgG	FITC	eBiosciences
F4/80	BM8	IgG2a	PE-Cy7	eBiosciences
CD4	GK1.5	IgG2b	FITC	eBiosciences
CD8	53-6.7	IgG2a	PE-Cy7	eBiosciences
CD25	PC61.5	IgG1	PerCP-Cy5.5	eBiosciences
FoxP3	FJK-16s	IgG2a	Alexa Fluor 700	eBiosciences
CD107a	1D4B	IgG2a	Alexa Fluor 488	eBiosciences
IFN-γ	MG-1.2	IgG1	APC	eBiosciences
IL-10	JES5-16E3	IgG2b	APC	eBiosciences
IL-12	C17.8	IgG2a	PE	eBiosciences

Table 5.1: FACS antibody list used in study.

Cells were washed and re-suspended in 200µL 3% formalin. To perform flow cytometric analyses a FACSLSRII 5 Laser (UV/Violet/Blue/Yellow-Green/Red) cytometer and BD Diva software (Becton Dickinson) were used. For each sample 50,000-200,000 events were recorded. Background staining was controlled by labelled isotype controls and fluorescence-minus-one. The results represent the percentage of positively stained cells in the total cell population exceeding the background staining signal. For determination of intracellular cytokine production by leukocytes, cells were incubated for 6 h at 37°C with BD Activation Cocktail plus GolgiPlug (PMA, Ionomycin and brefeldin A [BD Biosciences]); unstimulated controls were also set up for each cytokine study. Cells were then washed with staining buffer and stained at 4^{o}C for 30 min with appropriate surface mAb. Controls were stained with appropriate isotype-matched controls mAb. Cells were then fixed and saponin-permeabilised (Perm/fix solution, BD Biosciences) and incubated with mAb listed in Table S1 or isotype control mAb. After 30 min cells were twice washed in permealisation buffer (BD Biosciences) and then analysed by flow cytometry as described above. NK cells were identified as DX5^{+}/CD3^{-}, neutrophils as Ly6G^{+}, DC as CD11c^{+}, macrophages as F4/80^{+}, B cells as CD19^{+} and T cell as CD4^{+} and cytotoxic T cells as CD8^{+}.

5.4.7 Analysis of Cytokine Levels

Tumour and spleen homogenates were analysed using mouse pro-inflammatory 7-plex (IFN-γ, IL-1β, IL-6, IL-10, IL-12p70, KC/GRO/CINC, TNF-α; Meso Scale Discovery). All assays were performed per manufacturers' instructions. Cytokine levels are expressed as pg cytokine/ml (sensitivities of assays > 0.5-11pg/mL).

5.4.8 Evaluation of Antigen Specificity and Long-Term Tumour Protection

To test recall anti-tumour responses, naive and cured mice (primary B16F10 or CT26 tumour long-term; > 100 days) were rechallenged with the same tumourogenic dose of B16F10 or CT26 in the opposite flank and survival monitored over 100 days. Specifically, no further treatment was delivered following rechallenge. To assess the specificity of the pEEVGMCSF-B7.1 treatment was restricted to B16F10 or CT26 cells mice bearing two different tumour types, including lewis lung carcinoma and 4T1, were also assessed.

5.4.9 *In Vitro* Augmentation of Cytotoxic Activity

To assay cytotoxicity activity against CT26 and B16F10 tumour cells mixed splenocytes were harvested from mice responding to GMCSF-B7.1 treatment and resistant to tumour growth on rechallenge with B16F10 or CT26. The spleen was harvested and tumour-specific lymphocytes induced by incubating, 2 $x10^6$ splenocytes with 2 $x10^5$ mitomycin C-treated tumour cells in the presence of 25 IU/ml rmIL2 (sigma) for 5 days. Lymphoid cells were then harvested, washed three times in serum-free medium and applied as effectors at various effector: target ratios (100:1, 50:1, 1:1) with 2 $x10^4$ target cells. 50:1 ratio had optimum effects and therefore data is shown. Cells were incubated overnight in 96-well plates to allow target cell killing. Wells were then washed five times with PBS to eliminate non-adherent (dead and all effector cells). The MTT assay was used to quantify remaining living cells. MTT working solution (0.45mg/ml) was added to each well and cultures were incubated for 4 h at 37^{o}C. Culture medium was then removed from each well and the precipitate dissolved with 150ml of dimethyl sulfoxide (Sigma) for 10 min. Absorbance was read at 570nm and the percentage cytotoxicity was calculated.

5.4.10 Statistical Analysis

Experimental results were plotted and analysed for significance with Prism 4 software (GraphPad software Inc, CA, USA). $p<0.05$ was considered significant.

5.5 Results

5.5.1 Electroporation of pEEVGMCSF-B7.1 Results in Long-Term Inhibition of Tumour Growth in CT26 Murine Colorectal and B16F10 Metastatic Melanoma Models

In the current study, we investigated the therapeutic efficacy of pEEVGMCSF-B7.1 when compared to a standard vector also expressing GMCSF-B7.1. To test this, two tumour types (CT26 murine colorectal and B16F10 metastatic melanoma) were treated by electroporating tumours with pMG (standard plasmid backbone), pGT141GMCSF-B7.1 (standard plasmid therapy), pEEV (backbone) and pEEVGMCSF-B7.1 (Figure 5.2).

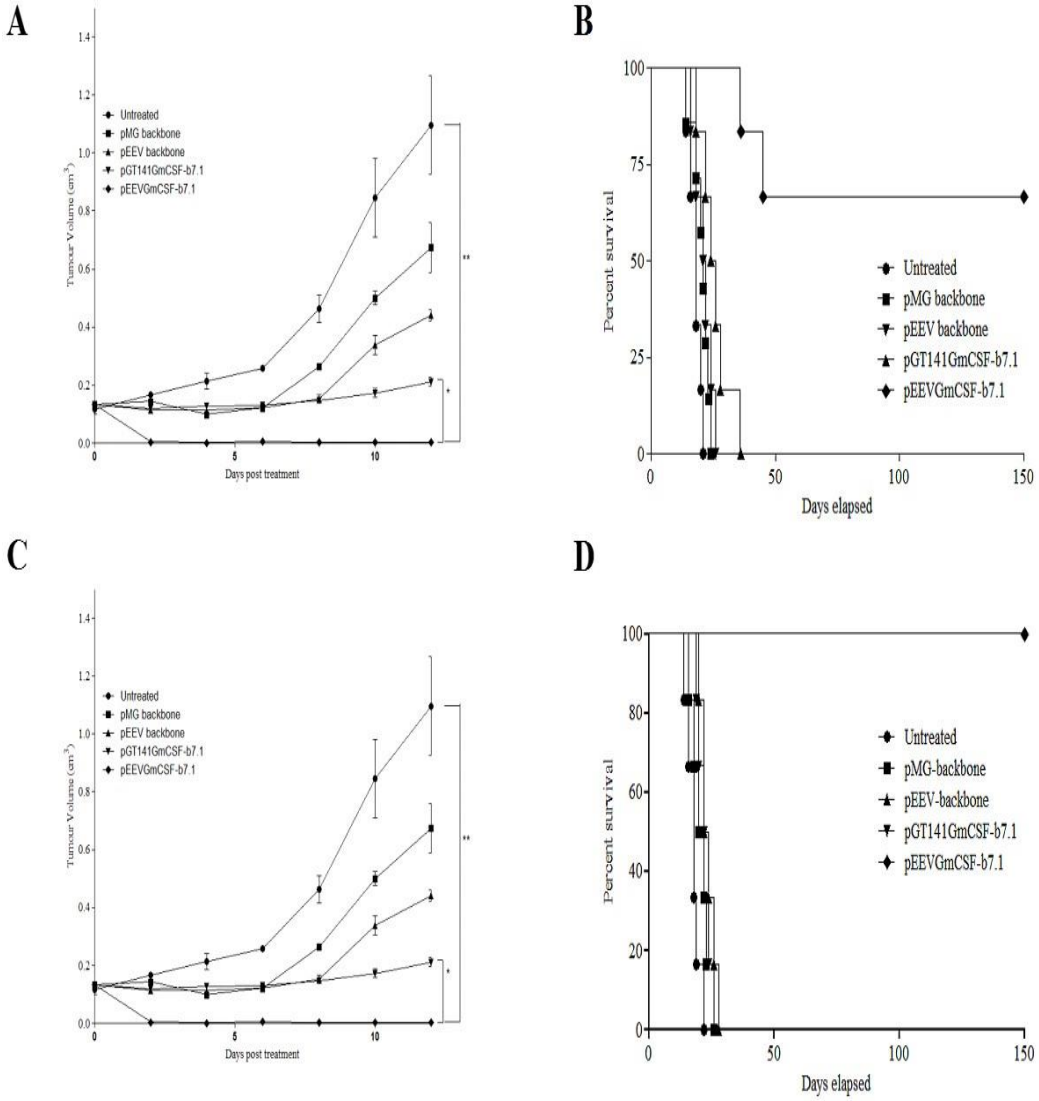
A
B
C
D
Untreated
pMG backbone
pEEV backbone
pGT141GmCSF-b7.1
pEEVGmCSF-b7.1
Tumour Volume (cm3)
Days post treatment
Percent survival
Days elapsed
pMG-backbone
pEEV-backbone

Figure 5.2: Therapeutic effect on established CT26 and B16F10 solid tumours. **(A)** Representative CT26 tumour growth curve: Each Balb/C mouse was subcutaneously injected with 1 $x10^6$ CT26 cells in the flank. On day 14 post tumour inoculation, tumours were treated with pMG (■), pGT141GMCSF-B7.1 (▲), pEEV (▼) and pEEVGMCSF-B7.1 (♦) or untreated (●). 6 mice/groups were used and the experiment was performed twice. Tumour volume was calculated using the formula $V=ab^2/6$. Data is presented as the means ± standard error of the mean. It was observed the pEEVGMCSF-B7.1 therapy delayed the growth of the tumours most effectively in comparison to the other groups. 17 days post treatment pEEVGMCSF-B7.1 significantly delayed tumour growth compared to untreated tumour (***p<0.0004) standard therapy vector pGT141GMCSF-B7.1 ** p<0.002. **(B)** Representative Kaplan-Meier survival curve of CT26 treated tumours was measured. Only mice treated with pEEVGMCSF-B7.1 survived. 66% of mice survived up to 150 days. All other groups were sacrificed by day 36 **(C)** Representative growth curve of B16F10 tumour. Each C57BL/6J was subcutaneously injected with 2 $x10^5$ B16F10 cells in the flank of the mice. On day 15 post tumour inoculation, tumours were treated with pMG (■), pGT141GMCSF-B7.1 (▲), pEEV (▼) and pEEVGMCSF-B7.1 (♦) or untreated (●), 6 mice/groups were used and the experiment was performed twice. 12 days post treatment pEEVGMCSF-B7.1 significantly delayed tumour growth compared to untreated tumour (**p<0.0001) standard therapy vector pGT141GMCSF-B7.1 (*p<0.0001). **(D)** Representative Kaplan-Meier survival curve of B16F10 showing pEEVGMCSF-B7.1 had 100% survival up to 150 days post treatment with all other groups sacrificed by day 28. Similar results were obtained in two independent experiments.

As expected the volumes of all non-electroporated (untreated) CT26 tumours and those treated with the empty plasmids, pMG and pEEV significantly increased (p<0.01) in size (Figure 5.2A). However, we did observe that the empty pEEV plasmid inhibited growth of the CT26 tumour between days 8-12, which we have observed previously [25]. Both therapeutic plasmids delayed the growth rate of the CT26 tumour. Importantly, the growth of pEEVGMCSF-B7.1 treated tumours was significantly more inhibited compared to pGT141GMCSF-B7.1 treated tumours (P<0.002) and untreated control tumours (p<0.0004). By day 26 post treatment, the untreated, pMG and pEEV treated groups were euthanized due to tumour size (Figure 5.2B). While the standard therapy pGT141GMCSF-B7.1 did inhibit tumour growth, all animals from this group were sacrificed by day 36 when the tumours reached the ethical size of 1.7cm^3. One mouse was removed from the pEEVGMCSF-B7.1 treated group on day 36 and again at day 45 due to tumours exceeding ethical size, however the remaining 66% survived up until day 150 post treatment when they were then sacrificed for subsequent immune analysis. To further test the efficacy of pEEVGMCSF-B7.1 therapy we utilised the B16F10 melanoma cell line due to its aggressive nature. Following the same experimental protocol as described for the CT26 model (Figure 5.2C) we again observed that untreated tumours grew exponentially with sacrifice from day 12 onwards (due to tumour size). Again we observed that pEEVGMCSF-B7.1 treatment delayed tumour growth when compared to the untreated group (p<0.0001) and pGT141GMCSF-B7.1 (p<0.0001). In terms of survival, the pMG, pEEV, untreated and pGT141GMCSF-B7.1 treated groups were sacrificed by day 28 (Figure 5.2D). Notably, we observed an even greater survival efficacy for the pEEVGMCSF-B7.1 (when compared to the CT26 model) in that 100 % mice survived and all remained tumour free for 150 days post treatment until they were removed for subsequent immune analysis. Similar results were obtained in both tumour types treated in a range of tumour sizes (approximately 100mm^3 to 350mm^3). Taken together, these data indicate that pEEVGMCSF-B7.1 treatment is able to significantly reduced (in the CT26 model) or prevent (B16F10 model) primary tumour growth.

5.5.2 Treatment with pEEVGMCSF-B7.1 Results in Robust Cellular Immune Recruitment in Systemic and Local Tumour Sites

As already indicated, for optimal cancer therapy, robust immune responses must be induced. Thus, to determine immune cell recruitment, we performed a comprehensive immune population profile of spleens and tumours 72 hour post treatment. CT26 tumour mice treated with pEEVGMCSF-B7.1 had a significantly greater percentage of splenic $CD19^+$ (B cells), $DX5^+/CD3^+$ (NKT cells), $DX5^+/CD3^-$ (NK cells) and $CD8^+$ (cytotoxic T cells) as shown in Table 5.2. Within the tumour environment we observed that the percentage of all cell types examined (with the exception of $CD4^+$ cells [T cells]) were significantly greater in pEEVGMCSF-B7.1 treated tumours when compared to untreated tumours. Importantly, when the therapeutic plasmid treatments were compared we observed that pEEVGMCSF-B7.1 treated mice had significantly more splenic and tumour $CD19^+$ cells ($P<0.05$) and significantly greater numbers of tumour $DX5^+/CD3^-$ ($p<0.001$) $F4/80^+$ (macrophages) ($P<0.001$) and $CD8^+$ ($P<0.001$) cells than control pGT141GMCSF-B7.1 treated mice. We observed a similar immune profile for the B16F10 treated mice (Figure. 5.3). Splenic and tumour $CD19^+$ ($p<0.001$), $DX5^+/CD3^+$ ($p<0.01$), $DX5^+/CD3^-$ ($p<0.01$), $CD11c^+$ (dendritic cells) ($p<0.001$), F4/80 ($p<0.001$) and $CD8^+$ ($p<0.001$) cells were all significantly higher for the pEEVGMCSF-B7.1 treated mice than untreated animals. However, we did not observe any significant differences in $CD4^+$ or TCR $\gamma\delta^+/CD3^+$ ($\gamma\delta$ T cells) [data not shown]. Notably, when the standard pGT141GMCSF-B7.1 therapy was compared to pEEVGMCSF-B7.1 the percentage of $CD19^+$ ($p<0.001$), $DX5^+/CD3^+$ ($p<0.01$), $CD11c^+$ ($p<0.001$) and $CD8^+$ ($p<0.001$) cells were all significantly greater indicating pEEVGMCSF-B7.1 recruits a superior immune recruitment locally at the tumour site. The spleen data had a similar trend as the tumour data with the percentage of $CD19^+$ ($p<0.01$), $DX5^+/CD3^+$ ($p<0.001$), $CD11c^+$ ($p<0.001$), F4/80 ($p<0.001$) and $CD8^+$ ($p<0.001$) cells all significantly greater in the pEEVGMCSF-B7.1 treated mice when compared to the standard therapy. These data indicate that treatment with pEEVGMCSF-B7.1 induces robust recruitment of innate and adaptive immune cell populations in both colorectal and metastatic melanoma models.

	Cell type	Tumour	Tissue	Untreated	pMG	pEEV	pGT141GmCSF-b7.1	pEEVGmCSF-b7.1
B Cells	$CD19^+$	CT26	Tumour	4.0 ± 0.6	5.3 ± 0.6	5.3 ± 0.9	6 ± 0.9	12.7 ± 1.2***/••
			Spleen	19.7 ± 2.5	25.6 ± 1.5	23 ± 3.3	36.3 ± 2.4	46.0 ± 2.4***/•
NK Cells	$DX5^+/CD3^+$		Tumour	2.2 ± 0.1	2.4 ± 0.3	2.2 ± 0.2	3.0 ± 0.6	6.4 ± 0.9**/••
			Spleen	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.1*/••
	$DX5^+/CD3^-$		Tumour	13.0 ± 1.0	10.7 ± 0.9	13.2 ± 0.6	11.8 ± 1.0	22.0 ± 2.0**/•••
			Spleen	2.5 ± 0.2	2.9 ± 0.4	2.9 ± 0.3	4.3 ± 0.6	6.1 ± 0.4
Dendritic cells	$CD11C^+$		Tumour	19.7 ± 0.7	23.3 ± 2.1	28.7 ± 2.7	30.4 ± 3.1	41.2 ± 2.3***
			Spleen	10.2 ± 0.2	9.6 ± 0.4	9.9 ± 1.1	9.1 ± 1.5	9.4 ± 1.3
Macrophages	$F4/80^+$		Tumour	13.1 ± 0.7	10.7 ± 0.5	12.4 ± 1.7	7.4 ± 1.5	21.4 ± 1.2*/•••
			Spleen	16.6 ± 0.7	15.5 ± 1.3	14.7 ± 1.8	12.3 ± 0.9	12.4 ± 1.6
	$CD4^+$		Tumour	4.5 ± 0.2	5.6 ± 0.5	6.4 ± 1.0	6.1 ± 0.4	5.2 ± 0.7
			Spleen	13.4 ± 2.3	14.6 ± 1.7	13.7 ± 1.5	14.5 ± 2.1	15.3 ± 1.5
CTL	$CD8^+$		Tumour	4.9 ± 0.4	5.7 ± 0.4	5.7 ± 0.8	7.6 ± 0.5	13.8 ± 1.1***/•••
			Spleen	4.0 ± 1.0	6.1 ± 0.1	5.2 ± 0.6	7.4 ± 0.6	10.1 ± 0.8**/•

Table 5.2: Percentage of immune cells in all tumour and spleen cells present 72 hours post treatment. Cells were isolated from CT26 tumours and spleens from treated and untreated control Balb/C mice. They were analysed by flow cytometry in which 20,000 events were recorded. Data represents the mean percentage of $CD19^+$ (B cells), $DX5^+/CD3^+$ (NKT cells), $DX5^+/CD3^-$ (NK cells), $CD11c^+$ (DC cells), $F4/80^+$ (Macrophage cells), $CD4^+$ and $CD8^+$ (T cells) positive cells at the time of analysis (48hours) post treatment. Data represents the mean percentage from 4 mice. The asterisks (*) indicate significant values of $*p<0.05$, $**p<0.01$, $***p<0.001$ as determined by one-way ANOVA following Bonferroni's multiple comparison of pEEVGMCSF-B7.1 compared to untreated tumour. The asterisks (●) indicate significance values of $^{\bullet}p<0.05$, $^{\bullet\bullet}p<0.01$, $^{\bullet\bullet\bullet}p<0.001$ as determined by one-way ANOVA following Bonferroni's multiple comparison of pEEVGMCSF-B7.1 compared to the standard vector pGT141GMCSF-B7.1.

	Cell type	Tumour	Tissue	Untreated	pMG	pEEV	pGT141GMCSF-B7.1	pEEVGMCSF-B7.1
B Cells	$D19^+$	B16F10	Tumour	12.5±2	8.8±2.4	20.3±1.9	26.1±3.1	54.1±2***/•••
			Spleen	46.6±3.1	46.7±2.3	51.9±2.9	53.3±1.6	68.7±1.4***/••
NK Cells	$DX5^+CD3^+$		Tumour	6.1±0.9	6.7±0.9	4.4±0.2	5.1±1.4	15.1±1.7**/••
			Spleen	1.0±0.1	1.2±0.1	1.1±0.1	1.4±0.2	4.7±0.1***/•••
	$DX5^+CD3^-$		Tumour	2.4±0.2	7.0±1.5	10.9±1.2	11.5±0.9	9.9±0.4**
			Spleen	2.4±0.6	2.8±0.4	3.3±0.1	4.8±0.1	5.7±0.4**
Dendritic cells	$CD11c^+$		Tumour	6.3±1.6	5.3±1.9	3.2±1.0	10.3±1.5	24.8±1.8***/•••
			Spleen	5.0±0.6	4.9±0.6	3.8±0.3	4.8±0.7	9.5±0.5***/•••
Macrophages	$F4/80^+$		Tumour	4.3±1.4	9.1±0.5	8.1±0.9	10.9±1.4	18.0±2.3***
			Spleen	9.9±0.7	11.5±1.0	8.3±0.6	11.5±0.3	16.0±0.9***/•••
	$CD4^+$		Tumour	4.3±1.0	1.7±0.7	7.7±1.2	7.5±1.5	9.7±1.3
			Spleen	20.9±1.1	22.9±1.6	19.2±0.4	18.5±0.9	18.6±1.1
CTL	$CD8^+$		Tumour	7.2±1.4	6.6±0.7	9.9±0.5	7.3±2.1	22.5±1.0***/•••
			Spleen	13.4±0.7	13.1±0.5	10.8±0.2	11.1±0.6	23.3±0.5***/•••

Table 5.3: Percentage of immune cells in all tumour and spleen cells present 72 hours post treatment. Cells were isolated from B16-F10 tumours and spleens from treated and untreated control C57Bl/6J mice. They were analysed by flow cytometry in which 20,000 events were recorded. Data represents the mean percentage of $CD19^+$ (B cells), $DX5^+/CD3^+$ (NKT cells), $DX5^+/CD3^-$ (NK cells), $CD11c^+$ (DC cells), $F4/80^+$ (Macrophage cells), $CD4^+$ and $CD8^+$ (T cells) positive cells at the time of analysis (48hours) post treatment. Data represents the mean percentage from 4 mice. The asterisks (*) indicate significant values of $*p<0.05$, $**p<0.01$, $***p<0.001$ as determined by one-way ANOVA following Bonferroni's multiple comparison of pEEVGMCSF-B7.1 compared to untreated tumour. The asterisks (●) indicate significance values of $•p<0.05$, $••p<0.01$, $•••p<0.001$ as determined by one-way ANOVA following Bonferroni's multiple comparison of pEEVGMCSF-B7.1 compared to the standard vector pGT141GMCSF-B7.1.

5.5.3 pEEVGMCSF-B7.1 Treatment Dampens Down Suppressive Treg Responses

T regulatory cells (Treg) are key to dampening effector cell responses, and therefore represent one of the main obstacles to effective anti-tumour responses. We therefore decided to analyse local Treg percentages in B16F10 tumours of treated animals (Figure 5.3). In the pEEVGMCSF-B7.1 treatment group (Figure 3.2B), $CD4^{+}CD25^{+}FoxP3^{+}$ (Treg) cell percentage was reduced significantly ($p<0.01$) compared to the untreated group (Figure 5.3A). Interestingly, we also observed a $CD4^{+}CD25^{-}FoxP3^{+}$ tumour cell population, which was also significantly reduced in the pEEVGMCSF-B7.1 treated animals when compared to untreated tumours ($p<0.01$), and also therapeutic control pGT141GMCSF-B7.1. These data show that electroporation with pEEVGMCSF-B7.1 reduces potentially detrimental suppressive tumour Treg populations.

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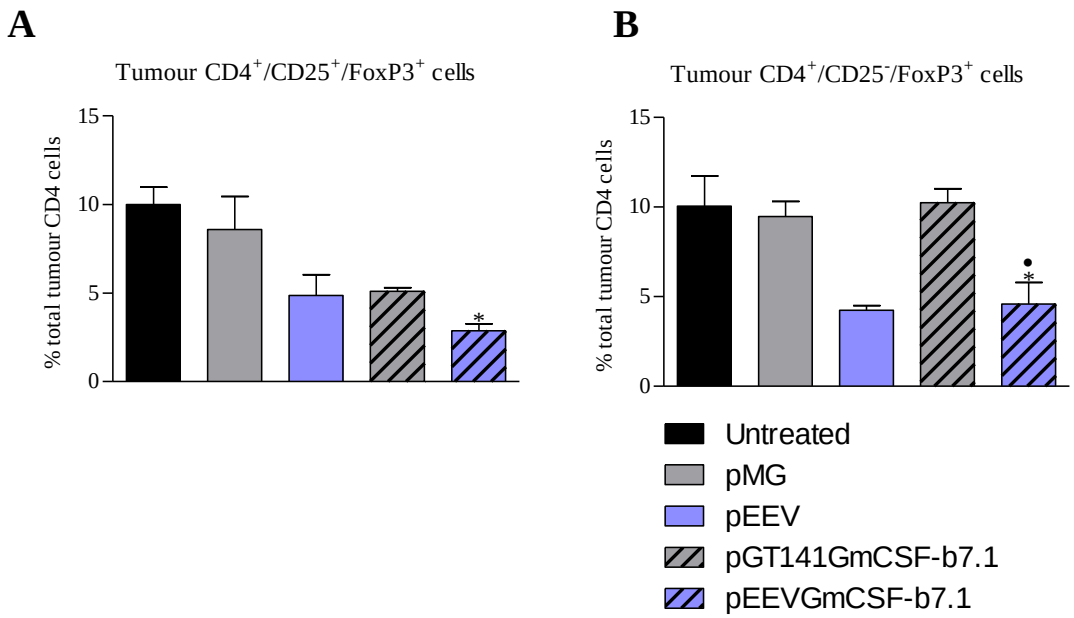


Figure 5.3: Percentage of the respective T cells found locally at the site of the B16F10 tumours treated with pMG, pEEV, pGT141GMCSF-B7.1, and pEEVGMCSF-B7.1 or untreated **(A)** Represents data obtained for the $CD4^+CD25^+FoxP3^+$cells **(B)** $CD4^+CD25^-FoxP3^+$ cells. Data represents the mean of the respective cells. Error bars show SD from 4 animals. The asterisks (*) indicate significant values of $*p<0.05$ as determined by one-way ANOVA following Bonferroni's multiple comparison of pEEVGMCSF-B7.1 compared to untreated tumour. The asterisks (●) indicate significance values of $^{\bullet}p<0.05$ as determined by one-way ANOVA following Bonferroni's multiple comparison of pEEVGMCSF-B7.1 compared to the standard vector pGT141GMCSF-B7.1. Similar results were obtained in two independent experiments.

5.5.4 Mice Receiving pEEVGMCSF-B7.1 Treatment Have a Pro-Inflammatory Cytokine Milieu in Spleens and Tumours

The cytokine milieu systemically and in the local tumour environment is often indicative of prognosis. Thus, we next examined the concentrations of cytokines within tumours and spleens in treated; untreated and healthy animals 72 hours post treatment (Figure 5.4**)**. Tumour and spleen concentrations of IFN-γ and IL-12 were all significantly elevated for the pEEVGMCSF-B7.1 treatments compared to untreated (p<0.001), pGT141GMCSF-B7.1 (p<0.001) and all other groups analysed. Tumour TNF-α levels were also significantly greater in pEEVGMCSF-B7.1 treated mice when compared to untreated (p<0.01), pGT141GMCSF-B7.1 (p<0.05) and all other groups analysed. In contrast, IL-10 levels were significantly reduced in pEEVGMCSF-B7.1 treated mice (both spleen and tumour) when compared to the pGT141GMCSF-B7.1 treated group (p<0.05) and all other groups analysed. Thus, pEEVGMCSF-B7.1 treatment appears to drive a strong inflammatory environment in both systemic and tumour sites, potentially via modulation of immune cell recruitment.

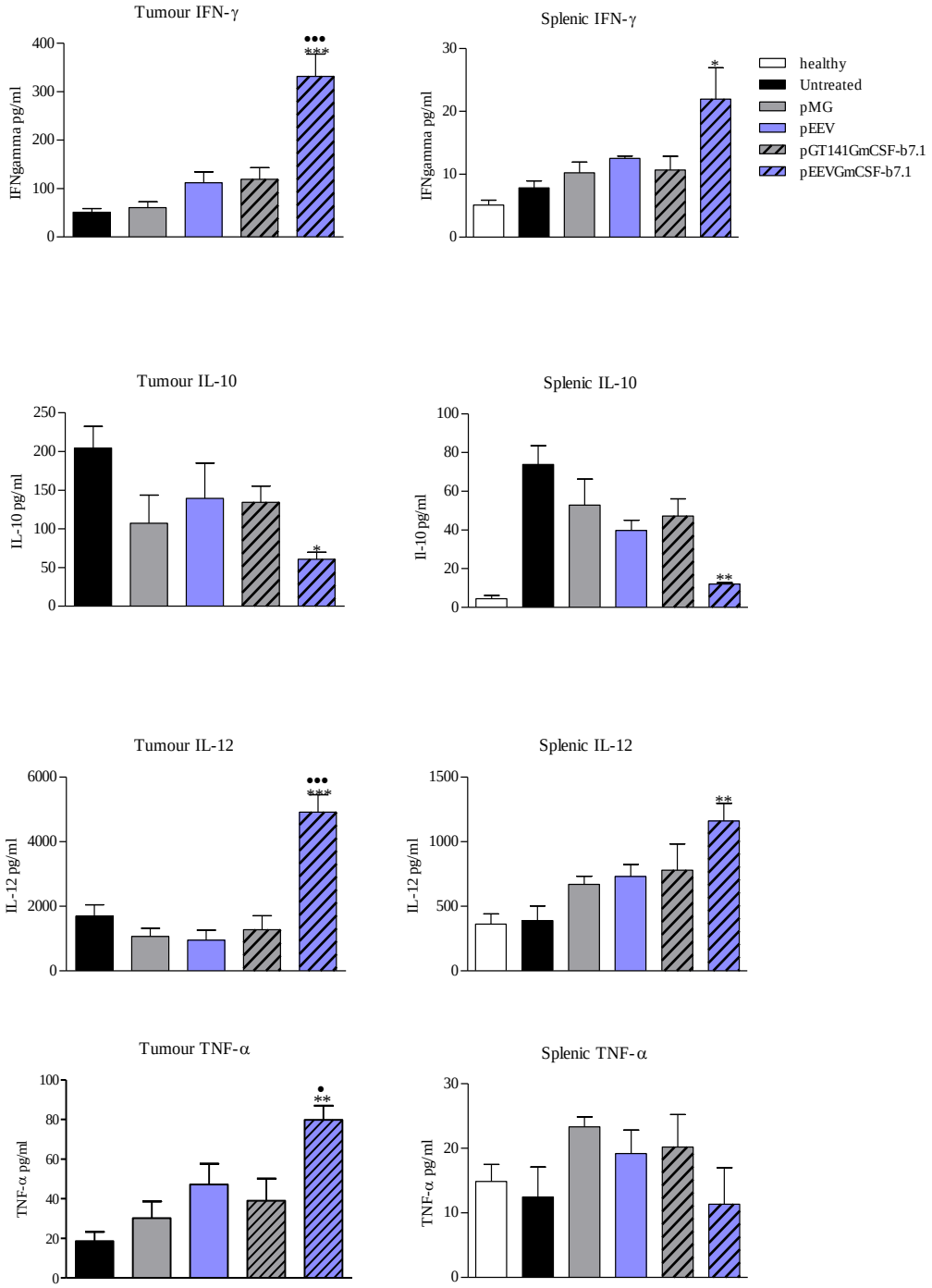


Figure 5.4: Cytokine levels (IFN-γ, IL-10, IL-12 and TNF-α) as measured from tumour and spleens isolated from B16F10 tumour challenged treated, untreated and healthy mice. The error bars represent the mean of 4 individual mice ± the SEM. The significance of differences was determined by one-way ANOVA following Bonferroni's multiple comparison (*p<0.05, ** p<0.01, *** p<0.001 untreated versus pEEVGMCSF-B7.1 and •p<0.05, ••p<0.01, •••p<0.001 pGT141GMCSF-B7.1 versus pEEVGMCSF-B7.1. Similar results were obtained in two independent experiments.

5.5.5 pEEVGMCSF-B7.1 Treated Mice Have Enhanced NK cell and B Cell Responses

NK cells have been shown to play critical roles in host immunity to cancer. As we observed a significant increase in this effector population in both systemic and tumour sites, we further characterised their response in B16F10 challenged mice. We observed that NK cells positive for IFN-γ were significantly higher ($p<0.001$) in the pEEVGMCSF-B7.1 treated groups when compared to untreated and pEEV control tumours (Figure 3.3**)**. We observed a similar trend in the splenic NK population with significantly elevated levels of IFN-γ positive NK cells compared to the untreated and healthy mice. Importantly when we analysed CD107a (LAMP-1), which is a sensitive marker of NK cell degranulation/cytotoxic activity, we observed significantly higher ($p<0.001$) levels of $CD107^+$ NK cells in pEEVGMCSF-B7.1 treated groups when compared to untreated and pEEV control tumours. The role of B cells in anti-tumour responses has been somewhat overlooked; however B cells can function as effector cells that mediate tumour immunity and destruction. Interestingly, we observed a significant increase in B cell percentages (both spleen and tumour; Table 5.2) and upon further investigation we noted that there was also a significant increase in B cells positive for IL-12 in pEEVGMCSF-B7.1 treated groups when compared to controls (Figure 5.5). These data indicate that pEEVGMCSF-B7.1 promotes the activation and function of NK cells and B cells during B16F10 challenge.

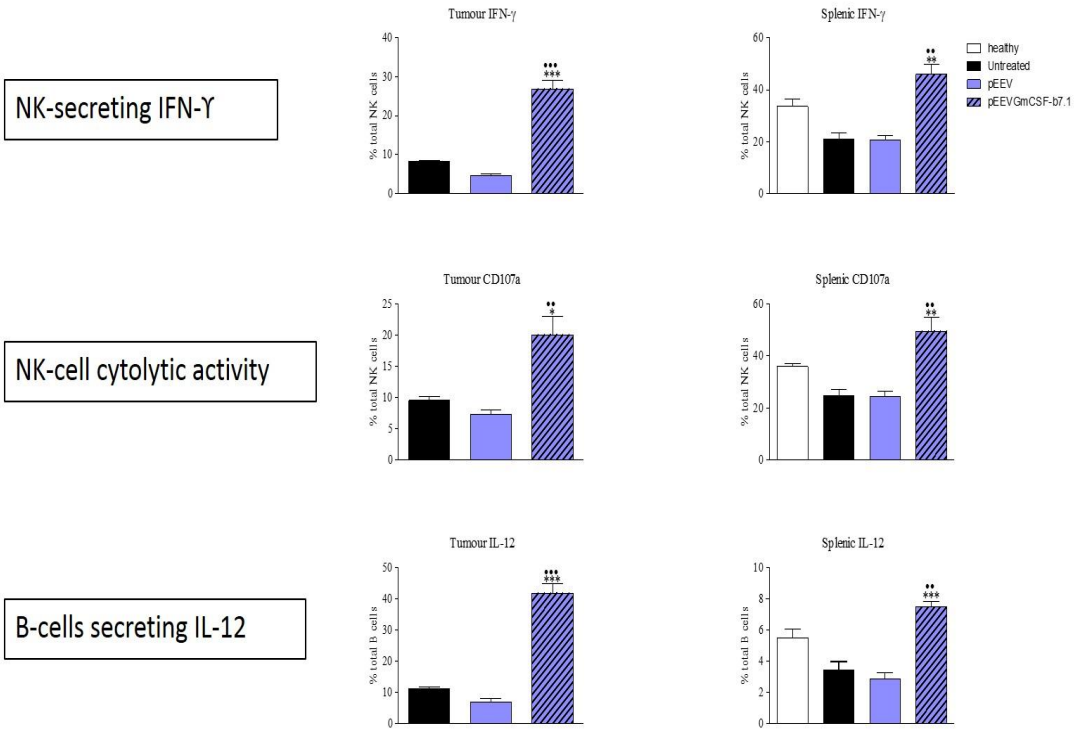


Figure 5.5: Cytotoxicity of NK and B cells in tumour and spleens of treated C57BL/6J mice. Data represents the mean of the respective cells. Error bars show SD from 4 animals. The asterisks (*) indicate significant values of *p<0.05, *p <0.01, ***p<0.001 as determined by one-way ANOVA following Bonferroni's multiple comparison pEEVGMCSF-B7.1 compared to untreated tumour. The asterisks (●) indicate significance values of ••p<0.01 and •••p<0.001 as determined by one-way ANOVA following Bonferroni's multiple comparison of pEEVGMCSF-B7.1 compared to the untreated groups. Similar results were obtained in two independent experiments.

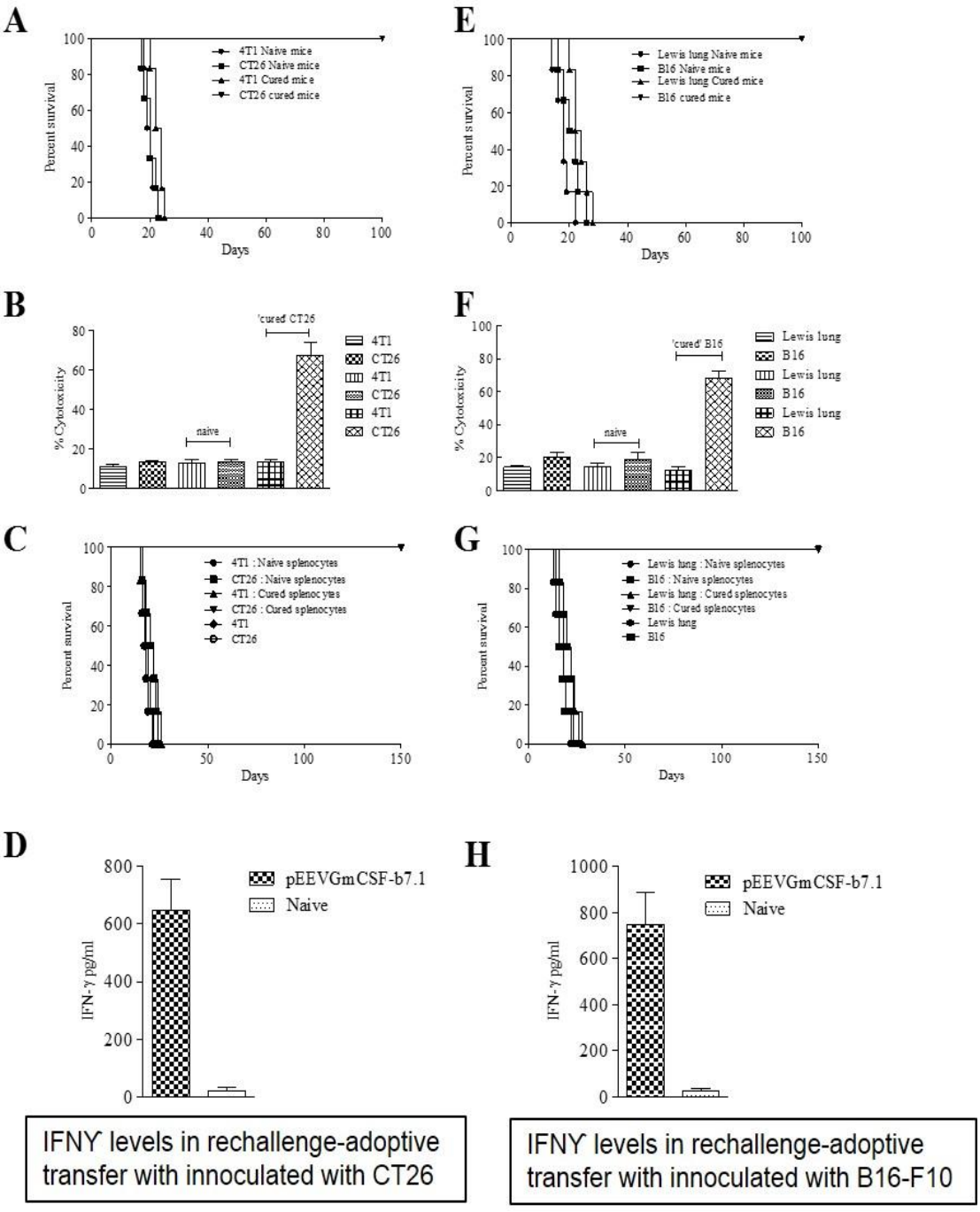
5.5.6 pEEVGMCSF-B7.1 Treatment Also Promotes Antigen-specific Secondary Tumour Protection Upon Re-challenge

There is strong evidence that links positive prognosis with robust tumour immune infiltrate in several different cancer types. As we observed a superior immune response after pEEVGMCSF-B7.1 treatment (Figure 5.3A and B), which was linked to reduced or absent primary tumours, we next challenged these mice (both CT26 and B16F10 cured and naive mice) to determine overall tumour protection. To compare tumour growth of 'cured mice', naive age-matched mice were inoculated with the same dose of viable tumour cells (Figure 5.6A and E). To determine tumour specific protection a different tumour was selected and cured and naive mice were challenged with either Lewis lung cancer (LLC) or Breast cancer (4T1). We observed long term (100 days) tumour-specific protection in pEEVGMCSF-B7.1 treated 'cured' mice group, both for the CT26 and B16F10 models. Notably, we observed that tumour protection was limited to the CT26 or B16F10 and not the previously unexposed tumours such as 4T1 and LLC in the respective models. This data suggests that pEEVGMCSF-B7.1 treatment results in a durable response.

To further confirm an antigen-specific response, we next determined the *in vitro* cytotoxicity of mixed splenic T lymphocyte population against CT26 and B16F10 cells. (Figure 5.6B and F). Cytotoxic responses of splenic T lymphocytes were significantly greater against CT26 and B16F10 cells from pEEVGMCSF-B7.1 treated 'cured' mice than in naive mice. To determine the specificity of this cytotoxicity we included the unexposed tumours 4T1 and LLC for the respective model. The splenic T lymphocytes against the CT26 and B16F10 demonstrated low percentage cytotoxicity. These results correspond with the observed immunity *in vivo* (Figure 5.6A and E).

The possible development of an immune mediated anti-tumour activity following pEEVGMCSF-B7.1 was further tested by a modified Winn assay (adoptive transfer), where groups received subcutaneous inoculation of a CT26 or B16F10 cell mixture and splenocytes from pEEVGMCSF-B7.1 treated 'cured' mice or naive mice, a mixture of 4T1 or Lewis lung cells and splenocytes from pEEVGMCSF-B7.1 treated 'cured' mice or naive mice, 4T1 or LLC and CT26 or B16F10 in their respective model (Figure 5.6C and G). All mice inoculated with splenocytes from naive mice developed tumours. Mice inoculated with mixtures of splenocytes and 4T1 or Lewis lung developed tumours, whereas no tumour growth

was observed in mice inoculated with splenocytes from pEEVGMCSF-B7.1 treated 'cured' mice in both the CT26 and B16F10 models indicating the protective effect was antigen specific as observed in the *in vitro* analysis. Control groups which were inoculated with CT26, B16F10, 4T1 or LLC cells all developed tumours and indicated that the tumours were growing in the correct manner. The tumour protective effect in the mice inoculated with splenocytes from pEEVGMCSF-B7.1 treated 'cured' mice in both the CT26 and B16F10 models resulted in prolonged survival (150 days). We also observed significantly high levels of IFN-γ from animals who received adoptively transferred mixtures of both CT26 and B16F10 and splenocytes from the pEEVGMCSF-B7.1 treated 'cured' mice of the respective model and naive mice of the same age (Figure 5.6D and H). This suggests adoptive transfer to naive mice of specific anti-tumour immune response provided protection to tumour challenge.



IFNY levels in rechallenge-adoptive transfer with innoculated with CT26

IFNY levels in rechallenge-adoptive transfer with innoculated with B16-F10

Figure 5.6: Tumour protection, cytotoxicity and immune memory. (**A)** Tumour protection was observed in the pEEVGMCSF-B7.1 treated CT26 mice when challenged (s.c.) with 1 $x10^6$ tumour cells (Balb/C n=6/group). 'Cured' and naive mice were challenged with CT26 and 4T1 tumour cells. Mice were monitored for tumour development. 100% survival was observed in the CT26 cured mice challenged with CT26. All other groups were sacrificed due to tumour burden by day 25. Similar results were obtained in two independent experiments. (**B)** Augmentation of the *in vitro* cytolytic activities of the spleen after pEEVGMCSF-B7.1 treatment of CT26 tumours. Groups included CT26, 4T1 cells, and Naive and 'CT26 cured' splenocytes incubated with CT26 and 4T1 cells respectively. The highest cytotoxicity was observed in the CT26 cells incubated with splenocytes obtained from 'CT26 cure' mice treated with pEEVGMCSF-B7.1. The data shown represents one of two separate experiments with similar results (n=6/group). (**C)** Adoptive transfer of lymphocytes of CT26 study. Mice (n=6) received subcutaneous injections of a mixture of mice receiving CT26 cells and splenocytes either from cured or naive mice, a mixture of 4T1 cells and splenocytes either from cured or naive mice, CT26 cells only or 4T1 cells only. All mice receiving mixtures of CT26 cells and splenocytes either from cured or pEEVGMCSF-B7.1 treatment survived up to 150 days whereas tumours developed in all animals within the other groups. (**D)** Interferon gamma production measured from supernatents obtained from stimulated splenocytes collected from re-challenged - adoptive transfer survivors (50 days post) and naive animals and IFN-γ was measured. High levels of IFN-γ were produced by pEEVGMCSF-B7.1 treated mice. The y-axis represents the concentration of IFN-γ in pg/ml of the supernatant from the stimulated splenocytes. Error bars show SD from 6 animals. (**E)** Tumour protection was observed in the pEEVGMCSF-B7.1 treated B16F10 mice when challenged (s.c.) with 2 $x10^5$ tumour cells (C57BL/6J n=6/group) in the left flanks. 'Cured' and naive mice were challenged with B16F10 and Lewis lung tumour cells. These mice were observed for tumour development. 100% survival was observed in the B16F10 cured mice challenged with B16F10. All other groups were sacrificed due to tumour burden by day 28. Similar results were obtained in two independent experiments. (**F)** Augmentation of the *in vitro* cytolytic activities of the spleen after pEEVGMCSF-B7.1 treatment of B16F10 tumours, the specific cytotoxicity was greatest at an effector target ratio of 50:1 after 48 hours incubation. Groups included B16F10, Lewis lung cells, and Naive and 'B16F10 cured' splenocytes incubated with B16F10 and Lewis lung cells respectively. The highest cytotoxicity was observed in the B16F10 cells incubated with splenocytes obtained from 'B16F10 cure' mice treated with pEEVGMCSF-B7.1. The data shown represents one of two separate experiments with similar results (n=6/group). (**G)** Adoptive transfer of lymphocytes of B16F10 study. Mice (n=6) received s.c. injections of a mixture of B16F10 cells and splenocytes either from cured or naive mice, a mixture of Lewis lung cells and splenocytes either from cured or naive mice, B16F10 cells only or Lewis lung cells only. All mice receiving mixtures of B16F10 cells and splenocytes either from cured from pEEVGMCSF-B7.1 treatment survived up to 150 days whereas tumours developed in all animals within the other groups. (**H)** Interferon gamma production measured from supernatents obtained from stimulated splenocytes collected from re-challenged - adoptive transfer survivors (approx 50 days post) and naive animals and IFN-γ was measured. High levels of IFN-γ were produced by pEEVGMCSF-B7.1 treated mice. The y-axis represents the concentration of IFN-γ in pg/ml of the supernatant from the stimulated splenocytes. Error bars show SD from 6 animals.

5.6 Discussion

Cancer treatment strategies utilising immunotherapy have recently gained clinical traction with the positive results emanating from Ipilimumab antibody clinical studies. The use of DNA plasmid based gene therapy for immunotherapy has also showed promise with encouraging data reported with IL-12 delivered via electroporation to patients with malignant melanoma [23].

We have developed a DNA plasmid which enables the enhanced expression of exogenous genes in transfected cancer cells. The pEEV was used to deliver via electroporation DNA encoding for GMCSF and B7.1 (pEEVGMCSF-B7.1) which we have demonstrated protects from primary and secondary tumour growth in both colon adenocarcinoma and melanoma cancer models by generating a robust pro-inflammatory immune cell recruitment and cytokine environment in systemic and tumour compartments.

Previously we have shown that delivery of our EEV plasmid (pEEV) via electroporation is capable of achieving reliable and superior expression in a variety of murine and porcine tissue types when compared to a control plasmid [25]. We therefore decided to engineer pEEV to contain the immune therapeutic genes; GMCSF and B7.1 and test efficacy in 2 aggressive cancer models. As already highlighted GMCSF and B7.1 are key molecules in inducing robust immune responses, which may facilitate subsequent anti-tumour immunity [26, 27]. Indeed, only those mice treated with pEEVGMCSF-B7.1 were observed to have reduced or absent primary tumour growth which paralleled to significantly improved survival rates. Mice treated with pEEVGMCSF and pEEVB7.1 delivered singly had minimal/no therapeutic effect (Data not shown). Correspondingly, when we analysed immune populations we observed that pEEVGMCSF-B7.1 treatment induced a significant increase in the levels of innate and adaptive immune cells both systemically and within the tumour environment. More specifically, increases in DC and macrophage levels were observed and may correspond to the presence of high GMCSF expression after pEEVGMCSF-B7.1 transduction, as previous studies have shown that $GMCSF^{-/-}$ mice have reduced DC and macrophage recruitment and survival [29, 30]. Several studies have also indicated that B cells can respond to GMCSF [31]. Within cancer immunology, B cells are currently under investigated, but appear to have a complex role. Interestingly, we observed that B cell populations

were increased after pEEVGMCSF-B7.1 treatment and upon further examination we observed that they had increased expression of IL-12 which correlated with total tissue levels of IL-12 in the spleen and tumour tissues of treated mice. IL-12 is a potent pro-inflammatory and anti-angiogenic cytokine capable of activating multiple aspects of innate and adaptive anti-tumour immunity, particularly via modulation of IFN-γ [32]. Indeed, we also observed increased tissue IFN-γ levels after pEEVGMCSF-B7.1 treatment, which potentially corresponds with the high levels of IFN-γ+ NK cells in pEEVGMCSF-B7.1 treated mice. Importantly IL-12 and indeed GMCSF are also able to regulate NK cell mediated cytotoxicity. NK cells are critical innate cells during cancer as they able to distinguish and destroy malignant from healthy cells, which is controlled by complex interactions of inhibitory and activating receptors which trigger specialised downstream effector signalling pathways [33, 34]. Importantly, we also observed a significant increase in NK cell levels and in tandem a high proportion expressing CD107a (cytotoxicity marker) in those mice treated with pEEVGMCSF-B7.1, highlighting the potential for pEEVGMCSF-B7.1 treatment to induce IL-12 expressing B cells which in turn potentially induces potent NK cell tumour killing and NK-derived IFN-γ production. Thus, these data emphasises the importance of the presence of these cell populations for cancer treatment and prognosis.

An overall reduction in tumour resident Tregs is indicated as a positive response for any cancer therapy [5]. Tregs cells are important regulators of immune cells and are seen as immune suppressors [5, 26]. Thus, any reduction of T09wq has the potential to allow immune cell recruitment, to the tumour site. pEEVGMCSF-B7.1 treatment correlates with Treg reduction with corresponding improved cellular infiltration. Interestingly, we also observed a $CD4^+CD25^-FoxP3^+$ population which was significantly reduced after pEEVGMCSF-B7.1 treatment. Previous studies have suggested that this population may act as a reservoir/precursor of $CD4^+CD25^+FoxP3^+$ Tregs, thus this reduction may assist in the reduced levels of Tregs present in our treated tumours [26]. Importantly, Treg cells also contribute to the production of immunoregulatory cytokines, such as IL-10 [35]. IL-10 is an immune-suppressing anti-inflammatory cytokine and is up regulated in many cancer models. Additionally, IL-10 down regulates the expression of T_H1 cytokines, such as IL-12 and IFN-γ and induces a Treg response [35, 36]. This reduced level of IL-10

observed in the pEEVGMCSF-B7.1 treated group correlates with the reduced levels of Treg cells and supports the positive inflammatory responses of this therapy.

The goal of all anti-cancer therapies is long-lasting responses that also prevent metastases and secondary tumours. As we observed such potent cellular and cytokine responses after pEEVGMCSF-B7.1 treatment we studied outcomes after tumour re-challenge. Notably, all naive mice succumbed to disease, while those in the pEEVGMCSF-B7.1 group developed no tumours suggesting protective and long-lasting immunity. As already discussed GMCSF is known to mature both DC and macrophages for antigen presentation. During our studies an antigen specific immune response was indeed suggested, as tumour protection and *in vitro* killing was limited to the CT26 or B16F10 and not to the previously unexposed tumours such as 4T1 and LLC in the respective models. Furthermore DCs and macrophages require help from NK cells for maturation, proper antigen presentation function and priming of T cell responses [37]. This bi-directional crosstalk during the early phases of tumour immunity as indicated by our data may also influence the following type and magnitude of adaptive immune response to pEEVGMCSF-B7.1. B cells are also important in antigen presentation to T cell populations. Indeed previous studies have indicated that activated B cells can be used as effective APCs for T-cell sensitisation to tumour antigens [38]. IL-10 has been shown to directly affect the function of antigen-presenting cells by inhibiting the expression of MHC and co-stimulatory molecules [39, 40], which in turn induces immune suppression or tolerance [41], thus reduced levels in to pEEVGMCSF-B7.1 treated mice may also correspond to robust antigen-specific immunity.

Although we did not investigate this mechanism of re-challenge protection in great detail (using mixed lymphocyte preps) we postulate that the anti-tumour generated immunity could be due to the action of a few different cell types. The protection could be antigen specific B cells producing cytokines that activate NK and T cell killing, and indeed a previous study has indicated that B cells may mediate tumour regression/protection after adoptive immunotherapy of solid tumours [42]. Finally, antigen-specific T cell responses, which are well known to be absolutely crucial for comprehensive anti-cancer immunity and eradication of tumours, would be a highly likely protective immune mechanism [5]. Clinically this protective nature is a very important observation. Recurring tumours post treatment is a major issue that exists with standard treatments like surgery with tumour cells left behind in

postoperative margins. This observation again supports the importance of this treatment and as its potential clinical adaption.

Importantly, the electrogene therapy with pEEVGMCSF-B7.1 protocol appears to be safe and non-toxic. All mice remained healthy throughout the course of the experiments and there were no treatment-related deaths. This suggests that electroporation of the solid tumours is well tolerated and the transgene expression did not induce systemic toxicity. Also, it was observed that long term survival of mice had no evidence of autoimmune disease, suggesting that, in this model, immune clearance of tumours could be achieved while maintaining autoimmune control.

Our findings suggest that the strategy of using pEEV as a therapeutic plasmid coding for two immunogenes, GMCSF and B7.1, in combination with electroporation compared to plasmids using the CMV promoter was far superior. The treatment established potent, durable tumour immunity and had a curative effect in two tumour models tested. As indicated above a robust immune response is important in cancer treatment outcome and prognosis. We demonstrated that pEEVGMCSF-B7.1 did induce a strong immune response, including enhanced B and NK cell responses. We can conclude that immunogene therapy of solid tumours by pEEV in combination of electroporation results in containment of the tumour. This strategy could be developed for clinical application.

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Chapter 6

General Discussion

In any disease condition, progress is dependent on an accurate diagnosis. If it were not possible to identify a disease, then the discovery of its causes, prediction of its outcomes, selection of appropriate treatments and the conduction of clinical trials becomes fraught with difficulty. When viewed in this context, cancer is a remarkably heterogeneous group of diseases.

For more than a century, we have classified cancers by their tissue or organ of origin e.g. breast/colon – and our therapies have been tailored accordingly. More recently, as our understanding of cancer cell biology has improved, we have developed a growing appreciation of cancer as a systemic disease [1, 2]. However, our improved understanding has been closely matched by cancer cell evolution [3-7]. Armed with the capacity for immune evasion, genetic instability and multidrug resistance [5-9], to name but a few, it is becoming increasingly clear that the future of anti-cancer therapies lies in a multi-modal approach.

In this thesis, we have adopted a "three-legged stool" approach to anti-cancer therapy. The first leg encompasses primary tumour ablation. Presently, tumour ablation is primarily reliant on surgical excision. However, while surgical resection has proven to be effective in the control of primary tumours and early-stage disease, a number of issues still need to be resolved. Advanced stage disease at presentation, anatomical locations that preclude complete excision as well as the presence of intercurrent illnesses all limit the application or adversely alter the risk-benefit profile of surgery [10-15]. Furthermore, although surgical resection confers statistically significant survival benefits over other therapeutic modalities, in the management of certain cancers such as cancers of the pancreas, lung and bronchus, it is still often associated with poor overall clinical outcomes [16-18].

To this extent, minimally invasive tumour ablation is a valuable clinical option in the anti-cancer arsenal. The efficiency of electroporation in tumour ablation has been reported by multiple investigators [19-21]. Clinical experience with reversible electroporation has been published extensively as a therapy that improves tissue permeability to specific chemotherapeutic agents providing local control of cutaneous cancers unsuitable for surgery and resistant to chemo/radiotherapy [22-32]. To date, the equipment available for electroporation limited its clinical utility to the treatment of cutaneous and subcutaneous tumour nodules. To address this need, we developed the EndoVe device, enabling endoscopic electroporation of gastrointestinal tissues and tumours. We conducted a pre-clinical evaluation of the

EndoVe prior to a phase I/II clinical study and validated the efficacy of the system in the treatment of cutaneous murine gastrointestinal tumours. In addition, we established the safety and utility of endoscopically-delivered electroporation through evaluation in a porcine model and by treating canine colorectal tumours.

The second leg of our stool involved immunotherapy – harnessing and enhancing the innate powers of the immune system to fight cancer. There are still many unmet needs in cancer, especially for patients with advanced, metastatic disease. In recent years, immunotherapies have succeeded in making significant advances in the treatment of patients with cancers previously considered incurable. The main types of immunotherapy presently employed in clinical practise include monoclonal antibodies, anticancer vaccines and non-specific immunotherapies.

Specifically, significant advances have been made in the management of malignant melanoma, known to be an immunogenic tumour. Between 2011 and now, the United States Food and Drug Authority (FDA) has approved seven novel agents, including Ipilimumab (anti-CTLA-4 antibody), Nivolumab (programmed death receptor-1 (PD-1) checkpoint inhibitor), Vemurafinib (BRAF-inhibitor) and Trametinib (MEK-inhibitor) intended to be used in advanced cases of melanoma. In phase II and III randomized trials on patients with previously treated metastatic disease, Ipilimumab, with or without a peptide vaccine, was found to improve overall survival [33-38]. More recently, Nivolumab, a programmed death receptor-1 (PD1-1) checkpoint inhibitor was shown either alone, or in combination with Ipilimumab, to be associated with significantly longer progression-free survival than Ipilimumab alone [39-42]. Moreover, a subset of patients with malignant melanoma has been found to carry the *c-Kit* mutation, traditionally associated with chronic myelogenous leukaemia. Early studies have demonstrated that treating this subset of patients with Imatinib, a tyrosine kinase inhibitor, demonstrated complete resolution of distant, established metastatic disease [43-48].

These immunologic advances are not limited to malignant melanoma alone. Prostate cancer was the first cancer type where the FDA approved the use of an anti-cancer vaccine, Sipuleucel-T based on observed survival benefits [49-51]. Furthermore, between a third and a quarter of patients with glioblastoma have been shown to carry the Epidermal Growth Factor Receptor vIII (EGFRvIII) mutation. Expression of EGFRvIII has been shown to correlate to increased tumourigenicity in mouse models and poor long term survival in clinical studies of patients. These

patients would traditionally have been treated with surgery followed by chemoradiotherapy. However, recent studies have demonstrated that vaccination with Rindopepimut has been shown to specifically eliminate cells expressing EGFRvIII. Rindopepimut stimulates the patient's immune system, inducing pronounced EGFRvIII-specific humoral and cellular responses. Phase II clinical trials have suggested that vaccination of newly diagnosed glioblastoma patients with Rindopepimut plus adjuvant granulocyte-macrophage colony-stimulating factor (GMCSF) resulted in prolonged progression-free and overall survival with minimal toxicity [52-56].

Many of the immune suppressive regulatory circuits that operate in tumours are part of the physiologic regulatory mechanisms used by the immune system to maintain homeostasis to prevent autoimmunity and temper inflammation after infection or injury [57-60]. Within this context, a number of studies exist describing the presence and accumulation of Treg in tumours. This accumulation increases with tumour volume and is coupled with the inhibition of innate immune rejection of the tumour and precludes the proliferation of effector cells leading to an overall poor prognosis [57-60]. Building on these observations we opted for a combination regime of Treg depletion with anti-CD25 antibodies and immunotherapy with the cytokine, pGMCSF-B7.1 While we observed a modest but significant improvement of primary tumour burden and survival for each individual treatment given alone or for the combination treated groups, the combination regime had the remarkable effect of eradicating pre-existing, established lung metastases when compared to the use of either treatment alone. This would indicate that the combination of Treg suppression with pGMCSF-B7.1 immunotherapy allowed for an enhanced anti-tumour CD8 immune response with the decrease in the levels of metastatic nodules. The potential clinical implications of this should not be understated as, for almost 2 centuries, scientists have been labouring to understand metastases, which account for nine out of ten cancer deaths, to finally disarm the latent enemy.

One of the principal challenges of immune therapy for cancer will be to establish novel treatments for those patients who do not carry the genetic mutations currently targeted by immune therapies and to minimise the side effect profile associated with the immune therapies which already exist [61-63]. While survival is the most important endpoint for evaluating cancer therapy, objective criteria based

on changes in the size of the tumour mass need to be developed in order to avert the danger of underestimating the efficacy of emerging immune therapies.

The third and final leg of our anti-cancer strategy involved the development of a safe, novel, non-viral enhanced expression vector system to facilitate the cytoplasmic delivery of therapeutic genes. Non-viral plasmid DNA gene therapy represents a promising approach for the treatment of many diseases including cancer. Intracellular delivery of DNA can be achieved with the application of electroporation, facilitating the initial transport of exogenous DNA across the hydrophobic cell membrane into the cytoplasm [19-21, 64]. Heretofore, the variable rates of subsequent transport of DNA from the cytoplasm to the nucleus for mRNA expression resulted in varying degrees of exogenous gene translation and proved a major limitation when compared to the high transfection rates associated with viral vectors. To overcome these expression difficulties, we developed a DNA-based enhanced expression vector (pEEV), with improved expression capabilities across a range of tissue histologies over standard non-viral DNA vectors. The anti-tumour effects of pEEV were demonstrated by the delayed growth and increased survival of the non-therapeutic pEEV-treated CT26 tumour model.

Having developed and established the DNA-based pEEV vector, we engineered pEEV to contain the immune therapeutic genes GMCSF and B7.1. The anti-tumour efficacy of electroporated pEEV plasmid, coding for GMCSF and the B7-1 co-stimulatory immune molecule (pEEVGMCSF-B7.1) in growing solid tumours was investigated and compared to a standard plasmid. pEEVGMCSF-B7.1 treatment was found to significantly enhance levels of both innate and adaptive immune populations in tumour and systemic sites, which corresponded to significantly increased tissue levels of pro-inflammatory cytokines including IFN-γ and IL-12. In contrast pEEVGMCSF-B7.1 treatment significantly reduced T regulatory populations and also the anti-inflammatory cytokine IL-10. Our data indicated that electro-immunogene therapy with the non-viral pEEVGMCSF-B7.1 was able to induce potent and durable anti-tumour immune responses that significantly reduce primary and also secondary tumour growth and thus represents a solid therapeutic platform for pursuing future clinical trials.

In this thesis, we demonstrated the viability and pre-clinical validation of EndoVe-mediated endoscopic electrochemotherapy as an alternative to surgery for primary tumour ablation. Furthermore, we demonstrated that while a combination of

Treg depletion and immunogenetherapy with GMCSF significantly delayed primary tumour growth, its most significant effect was in the eradication of pulmonary metastases in the B16-F10 murine melanoma model. This is significant for two reasons. Firstly, the B16-F10 melanoma model is known to be a poorly immunogenic, highly aggressive model for murine tumour immunotherapy studies [65]. Additionally, while primary tumours are often amenable to surgical excision or debulking, 9 out of 10 patients with melanoma die from their metastatic disease burden. In Chapter 4, we developed a novel, non-viral pEEV vector system, achieving mRNA expression levels far superior to those observed using conventional plasmid DNA vectors. Following on from this observation, we then utilised this pEEV vector system in combination with the immune cytokine GMCSF-B7.1 leading to robust recruitment of immune effector cells with consequent potent, durable and transferable tumour antigen-specific responses.

We would foresee the future directions of this thesis incorporating expansion of the use of endoscopic electrochemotherapy to target a variety of intraluminal tissues and tumours. Furthermore, novel therapies are needed for patients who do not carry the genetic mutations currently targeted by immune therapies. In addition, coupling of electrochemotherapy with immune-potentiating agents such as checkpoint blockade ligation or iCos agonists may facilitate enhanced anti-tumour responses.

When President Richard Nixon declared war on cancer in 1971, no one could have imagined the true magnitude of the undertaking. Some four decades on, we have made meaningful strides in the battle against this mortal enemy, yet millions still die from cancer each year. It is my belief that we are seeing a paradigm shift in cancer therapy, where the focus has moved to targeting and harnessing the immune system as opposed to targeting the tumour itself. Characterisation of the molecular identities of a number of tumour-associated antigens has provided a major stimulus for the development of new immunotherapies for the treatment of patients with solid cancers. However, we need to be cautious about coalescing anecdotal stories of success into data.

It is my sincere hope that we may be the generation to finally weed out the cancer cell from its reluctant host. However, it is critical that when we explore and shape this dialogue on cancer, we do not lose sight of the broader view of health, incorporating quality of life, the sociological and psychological impact of this

tremendous disease on the patient and their families, and the interplay of other co-morbidity with cancer.

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